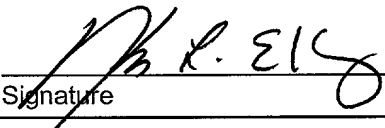


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Population	1,000,000	1,050,000	1,100,000	1,150,000	1,200,000	1,250,000	1,300,000	1,350,000	1,400,000	1,450,000	1,500,000	1,550,000	1,600,000	1,650,000	1,700,000	1,750,000	1,800,000	1,850,000	1,900,000	1,950,000	2,000,000	2,050,000	2,100,000	2,150,000	2,200,000	2,250,000	2,300,000	2,350,000	2,400,000	2,450,000	2,500,000	2,550,000	2,600,000	2,650,000	2,700,000	2,750,000	2,800,000	2,850,000	2,900,000	2,950,000	3,000,000	3,050,000	3,100,000	3,150,000	3,200,000	3,250,000	3,300,000	3,350,000	3,400,000	3,450,000	3,500,000	3,550,000	3,600,000	3,650,000	3,700,000	3,750,000	3,800,000	3,850,000	3,900,000	3,950,000	4,000,000	4,050,000	4,100,000	4,150,000	4,200,000	4,250,000	4,300,000	4,350,000	4,400,000	4,450,000	4,500,000	4,550,000	4,600,000	4,650,000	4,700,000	4,750,000	4,800,000	4,850,000	4,900,000	4,950,000	5,000,000	5,050,000	5,100,000	5,150,000	5,200,000	5,250,000	5,300,000	5,350,000	5,400,000	5,450,000	5,500,000	5,550,000	5,600,000	5,650,000	5,700,000	5,750,000	5,800,000	5,850,000	5,900,000	5,950,000	6,000,000	6,050,000	6,100,000	6,150,000	6,200,000	6,250,000	6,300,000	6,350,000	6,400,000	6,450,000	6,500,000	6,550,000	6,600,000	6,650,000	6,700,000	6,750,000	6,800,000	6,850,000	6,900,000	6,950,000	7,000,000	7,050,000	7,100,000	7,150,000	7,200,000	7,250,000	7,300,000	7,350,000	7,400,000	7,450,000	7,500,000	7,550,000	7,600,000	7,650,000	7,700,000	7,750,000	7,800,000	7,850,000	7,900,000	7,950,000	8,000,000	8,050,000	8,100,000	8,150,000	8,200,000	8,250,000	8,300,000	8,350,000	8,400,000	8,450,000	8,500,000	8,550,000	8,600,000	8,650,000	8,700,000	8,750,000	8,800,000	8,850,000	8,900,000	8,950,000	9,000,000	9,050,000	9,100,000	9,150,000	9,200,000	9,250,000	9,300,000	9,350,000	9,400,000	9,450,000

Small Entity Statement, which is: <input type="checkbox"/> Unsigned; <input type="checkbox"/> Newly signed for this application; <input type="checkbox"/> A copy from prior application [**SERIAL NUMBER**] and such small entity status is still proper and desired.	0 pages
Preliminary Amendment	0 pages
IDS	0 pages
Form PTO 1449	0 pages
Cited References	0 pages
Recordation Form Cover Sheet and Assignment	0 pages
Assignee's Statement	0 pages
English Translation	0 pages
Certified Copy of Priority Document	0 pages
Return Receipt Postcard	1
<b>FILING FEES:</b>	
Basic Filing Fee: \$760/\$380	\$ 380.00
Excess Claims Fee: 35 - 20 x \$9/\$18	\$ 135.00
Excess Independent Claims Fee: 11 - 3 x \$78/\$39	\$ 312.00
Multiple Dependent Claims Fee: \$260/\$130	\$ 130.00
Total Fees:	\$ 957.00
<input checked="" type="checkbox"/> Enclosed is a check for \$957.00 to cover the total fees. <input type="checkbox"/> Charge [**AMOUNT**] to Deposit Account No. 03-2095 to cover the total fees. <input type="checkbox"/> The filing fee is not being paid at this time. <input checked="" type="checkbox"/> Please apply any other charges, or any credits, to Deposit Account No. 03-2095.	
<b>CORRESPONDENCE ADDRESS:</b>	
Karen L. Elbing, Ph.D. Reg. No. 35,238 Clark & Elbing LLP 176 Federal Street Boston, MA 02110	
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Sandra E. Marxen

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Signature of person mailing correspondence

APPLICANT : GARY RUVKUN AND SCOTT OGG

TITLE : THERAPEUTIC AND DIAGNOSTIC TOOLS FOR IMPAIRED GLUCOSE TOLERANCE CONDITIONS

THERAPEUTIC AND DIAGNOSTIC TOOLS  
FOR IMPAIRED GLUCOSE TOLERANCE CONDITIONS

5                   Statement as to Federally Sponsored Research

This invention was made in part with support from the Federal government through NIH Grant Nos. AG05790 and AG14161. The Government has certain rights in the invention.

Background of the Invention

10               This invention relates to compositions and methods useful for delaying or ameliorating human diseases associated with glucose intolerance.

This application is a continuation-in-part of PCT/US98/10080, filed May 15, 1998, which is a continuation-in-part of U.S.S.N. 08/888,534, filed July 7, 1997, and U.S.S.N. 08/857,076, filed May 15, 1997.

15               Diabetes is a major disease affecting over 16 million individuals in the United States alone at an annual cost of over 92 billion dollars. Type I diabetes or insulin-dependent diabetes (IDDD) is an autoimmune disease. In the IDDM patient, the immune system attacks and destroys the insulin-producing beta cells in the pancreas. The central role of insulin in human metabolism is to aid in the  
20               transport of glucose into muscle cells and fat cells. The body's inability to produce insulin results in hyperglycemia, ketoacidosis, thirst, and weight loss. In addition, diabetics often suffer from chronic atherosclerosis and kidney and eyesight failure. A patient with IDDM requires daily injections of insulin to

survive.

The most common form of diabetes is non-insulin dependent diabetes (NIDDM) or Type II diabetes. Type II diabetes is a heterogenous group of disorders in which hyperglycemia results from both impaired insulin secretory  
5 response to glucose and decreased insulin effectiveness (i.e., insulin resistance). Older people who are overweight are at particular risk for Type II diabetes. Genetic studies have suggested that, Type II diabetes is found in families and that the disease may be due to multiple genetic defects. In addition, the link between obesity and Type II diabetes is strong. Approximately 80 percent of Type II  
10 diabetics are obese. Weight loss and exercise can be effective to keep blood glucose levels normal, reducing the long-term complications of the disease.

At present there are few reliable methods for presymptomatic diagnosis of a genetic predisposition for diabetes or obesity. The search for genetic markers linked to diabetes and obesity has proven difficult, and this is especially true for  
15 Type II diabetes.

Treatments for diabetes emphasize control of blood glucose through blood glucose monitoring. The majority of patients take oral medications and/or insulin injections for appropriate control. Treatment of diabetes is generally chronic and lifelong, and treatments are generally not satisfactory over the long  
20 run. In addition, insulin treatment may become increasingly ineffective as the disease progresses. While insulin has been known for decades, and within the past decade, the receptors for insulin and aspects of its signaling pathway have been identified, the transcriptional output from these signaling pathways have not been characterized. In addition, the molecular basis of the obesity-induced insulin  
25 resistance is unknown.

### Summary of the Invention

We have discovered that the *C. elegans* metabolic regulatory genes *daf-2* and *age-1* encode homologues of the mammalian insulin receptor/PI 3-kinase signaling pathway proteins, respectively. We have also discovered that the *C. elegans* PKB kinase and AKT kinase act downstream of these genes, as their mammalian homologues act downstream of insulin signaling. These results further endorse the congruence between the *C. elegans* and mammalian insulin signaling pathways, strongly supporting the contention that new genes identified in the *C. elegans* pathway also act in mammalian insulin signaling. In addition, we have also found that the *C. elegans* PTEN lipid phosphatase homologue, DAF-18, acts upstream of AKT in this signaling pathway. Thus, our molecular genetic analysis maps mammalian PTEN action to the insulin signaling pathway.

We have also discovered that the DAF-16 forkhead protein represents the major transcriptional output of this insulin signaling pathway. For example, we have discovered that it is the dysregulation of the DAF-16 transcription factor in the absence of insulin signaling that leads to metabolic defects; inactivation of DAF-16 reverses the metabolic defects caused by lack of insulin signaling in *C. elegans*. We have found 3 human DAF-16 orthologues: FKHL1, FKHL, and AFX. Our molecular genetic analysis strongly suggests that the activity of these transcription factors is strongly coupled to insulin signaling and that drug-induced inhibition of one or all of these transcription factors ameliorates diabetic disease. As discussed in more detail below, we have developed screening strategies to identify such drugs.

We have also found that the *C. elegans* *daf-7*, *daf-1*, *daf-4*, *daf-8*, *daf-14*, and *daf-3* genes encode neuroendocrine/target tissue TGF- $\beta$  type signal

transduction molecules that genetically interact with the insulin signaling pathway. Similarly, we have shown that the metabolic defects caused by lack of neuroendocrine TGF- $\beta$  signals can be reversed by inactivation of the DAF-3 transcription factor. Finally, we have found that another *C. elegans* gene, *daf-18*,  
5 the homologue of the mammalian PTEN lipid phosphatase gene, also functions in the DAF-2 signaling pathway.

Together, this evidence indicates that the DAF-16, DAF-3, DAF-8, and DAF-14 transcriptional outputs of these converging signaling pathways regulate metabolism. In addition, these discoveries also indicate that insulin and TGF-  
10  $\beta$ -like signals are integrated in humans to regulate metabolism, and that the homologues of other DAF proteins are likely to be defective or down regulated in human diabetic pedigrees as well as obesity induced diabetes. These results therefore indicate that the *C. elegans daf* genes are excellent candidate genes and proteins for human disease associated with glucose intolerance, e.g., diabetes,  
15 obesity, and atherosclerosis. Our findings indicate that the human homologues of these *daf* genes and proteins mediate insulin signaling in normal people and may be defective or mis-regulated in diabetics. Moreover, our findings indicate that there are at least two classes of type II diabetics: those with defects in the TGF- $\beta$  signaling genes, and those with defects in insulin signaling genes. Below we  
20 describe exemplary sequence and functional characteristics of the human homologues of the *daf* genes.

The discovery of converging DAF-7 and DAF-2 insulin-like signaling indicates that many cases of obesity-induced and genetically-induced diabetes (and obesity) may be treated by administration of a human DAF-7 polypeptide.

25 The discovery that defects in the TGF- $\beta$  signaling pathway can be

suppressed by decreases in DAF-3 pathway activity and that defects in the insulin pathway can be suppressed by decreases in DAF-16 activity highlight the utility of transcriptional regulatory DAF proteins in drug development; in particular, drugs that inhibit the activity of these proteins are useful for reversing the effects of obesity-induced or genetically-induced defects in DAF-7 TGF- $\beta$  type or insulin signaling.

In one aspect, the invention features a substantially pure preparation of a DAF-2 polypeptide, which can be derived from an animal (for example, a mammal, such as a human, or an invertebrate, such as *C. elegans*). In preferred embodiments, the DAF-2 polypeptide has insulin receptor (InR) activity, insulin receptor related activity, insulin-like growth factor receptor (IGF-1) receptor activity, or a combination of these activities.

The invention also features isolated DNA encoding a DAF-2 polypeptide. This isolated DNA can have a nucleotide sequence that includes, for example, the nucleotide sequence of the *daf-2* gene shown in Fig. 2B. This isolated DNA can also, in preferred embodiments, complement a *daf-2* mutation in *C. elegans*, an InR mutation in a mouse, or an IGF-1 receptor mutation in a mouse.

The isolated DNA encoding a DAF-2 polypeptide can be included in a vector, such as a vector that is capable of directing the expression of the protein encoded by the DNA in a vector-containing cell. The isolated DNA in the vector can be operatively linked to a promoter, for example, a promoter selected from the group consisting of *daf-2*, *age-1*, *daf-16*, *daf-1*, *daf-4*, *daf-3*, and *akt* promoters. The isolated DNA encoding a DAF-2 polypeptide, or a vector including this DNA, can be contained in a cell, such as a bacterial, mammalian, or nematode cell.

Also included in the invention is a method of producing a recombinant

DAF-2 polypeptide, and a DAF-2 polypeptide produced by this method. This method involves (a) providing a cell transformed with isolated DNA that (i) encodes a DAF-2 polypeptide, and (ii) is positioned for expression in the cell, under conditions for expressing the isolated DNA, and (b) isolating the  
5 recombinant DAF-2 polypeptide.

A substantially pure antibody, such as a monoclonal or polyclonal antibody, that specifically recognizes and binds a DAF-2 polypeptide is also included in the invention.

The invention also features a method of detecting a gene, or a portion of  
10 a gene, that is found in a human cell and has sequence identity to the *daf-2* sequence of Fig. 2B. In this method, isolated DNA encoding a DAF-2 polypeptide, a portion of such DNA greater than about 12 residues in length, or a degenerate oligonucleotide corresponding to SEQ ID NOS: 33, 34, 79, 80, 81, 82, 83, or 84, is contacted with a preparation of DNA from the human cell under  
15 hybridization conditions that provide detection of DNA sequences having about 70% or greater nucleic acid sequence identity to the *daf-2* sequence of Fig. 2B. This method can also include a step of testing the gene, or portion thereof, for the ability to functionally complement a *C. elegans daf-2* mutant.

Another method included in the invention is a method of isolating a  
20 gene, or a portion of a gene, that is found in a human cell and has at least 90% nucleic acid sequence identity to a sequence encoding SEQ ID NOS: 33, 34, 79, 80, 81, 82, 83, or 84. This method involves (a) amplifying by PCR the human gene, or portion thereof, using oligonucleotide primers that (i) are each greater than about 12 residues in length, and (ii) each have regions of complementarity to  
25 opposite DNA strands in a region of the nucleotide sequence of Fig. 2B, and (b)

isolating the human gene, or portion thereof. This method can also include a step of testing the gene, or portion thereof, for the ability to functionally complement a *C. elegans daf-2* mutant.

5 In another aspect, the invention features a substantially pure preparation of a DAF-3 polypeptide, which can be derived from an animal (for example, a mammal, such as a human, or an invertebrate, such as *C. elegans*). In a preferred embodiment, the polypeptide is a SMAD protein. In other preferred embodiments, the polypeptide is capable of binding and interacting with a nematode DAF-1, DAF-4, DAF-8, DAF-14, or DAF-16 polypeptide.

10 The invention also features isolated DNA encoding a DAF-3 polypeptide. This isolated DNA can have a sequence that includes, for example, the nucleotide sequence of a *daf-3* gene shown in Figs. 11A, 11B, or 11C. This isolated DNA can also, in preferred embodiments, complement a *daf-3* mutation in *C. elegans* or complement a *daf-3* knockout mouse.

15 The isolated DNA encoding a DAF-3 polypeptide can be included in a vector, such as a vector that is capable of directing the expression of the protein encoded by the DNA in a vector-containing cell. The isolated DNA in the vector can be operatively linked to a promoter, for example, a promoter selected from the group consisting of *daf-3*, *daf-4*, *daf-16*, *daf-2*, *age-1*, and *akt* promoters. The  
20 isolated DNA encoding a DAF-3 polypeptide, or a vector including this DNA, can be contained in a cell, such as a bacterial, mammalian, or nematode cell.

Also included in the invention is a method of producing a recombinant DAF-3 polypeptide, and a DAF-3 polypeptide produced by this method. This method involves (a) providing a cell transformed with isolated DNA that (i)  
25 encodes a DAF-3 polypeptide, and (ii) is positioned for expression in the cell,

under conditions for expressing the isolated DNA, and (b) isolating the recombinant DAF-3 polypeptide.

A substantially pure antibody, such as a monoclonal or polyclonal antibody, that specifically recognizes and binds a DAF-3 polypeptide is also  
5 included in the invention.

The invention also features a method of detecting a gene, or a portion of a gene, that is found in a human cell and has sequence identity to any of the *daf-3* sequences of Figs. 11A, 11B, or 11C. In this method, isolated DNA encoding a DAF-3 polypeptide, a portion of such DNA that is greater than about 12 residues  
10 in length, or a degenerate oligonucleotide corresponding to SEQ ID NOS: 35, 36, or 85, is contacted with a preparation of DNA from the human cell under hybridization conditions that provide detection of DNA sequences having about 70% or greater nucleic acid sequence identity to any of the *daf-3* sequences of Figs. 11A, 11B, or 11C. This method can also include a step of testing the gene, or  
15 portion thereof, for the ability to functionally complement a *C. elegans daf-3* mutant.

Another method included in the invention is a method of isolating a gene, or a portion thereof, that is found in a human cell and has at least 90% nucleic acid sequence identity to a sequence encoding SEQ ID NOS: 35, 36, or 85.  
20 This method includes (a) amplifying by PCR the human gene, or portion thereof, using oligonucleotide primers that (i) are each greater than about 12 residues in length, and (ii) each have regions of complementarity to opposite DNA strands in a region of any of the nucleotide sequences of Figs. 11A, 11B, or 11C, and (b) isolating the human gene, or portion thereof. This method can also include a step  
25 of testing the gene, or portion thereof, for the ability to functionally complement a

*C. elegans daf-3* mutant.

In yet another aspect, the invention features a substantially pure preparation of DAF-16 polypeptide, which can be derived from an animal (for example, a mammal, such as a human, or an invertebrate, such as *C. elegans*). In a preferred embodiment, the polypeptide is a forkhead transcription factor that binds DNA. In other preferred embodiments, the polypeptide is capable of interacting with a polypeptide selected from the group consisting of DAF-3, DAF-8, and DAF-14.

The invention also features isolated DNA encoding a DAF-16 polypeptide. This isolated DNA can have a sequence that includes, for example, the sequence of the *daf-16* gene shown in Figs. 13A or 13B. This isolated DNA can also, in preferred embodiments, complement a *daf-16* mutation in *C. elegans*, or complement an FKHR, FKHRL1, or AFX mutation in a mouse.

The isolated DNA encoding a DAF-16 polypeptide can be included in a vector, such as a vector that is capable of directing the expression of the protein encoded by the DNA in a vector-containing cell. The isolated DNA in the vector can be operatively linked to a promoter, for example, a promoter selected from the group consisting of *daf-2*, *age-1*, *daf-16*, *daf-3*, *daf-4*, and *akt* promoters. The isolated DNA encoding a DAF-16 polypeptide, or a vector containing this DNA, can be contained in a cell, such as a bacterial, mammalian, or nematode cell.

Also included in the invention is a method for producing a recombinant DAF-16 polypeptide, and a DAF-16 polypeptide produced by this method. This method involves (a) providing a cell transformed with purified DNA that (i) encodes a DAF-16 polypeptide, and (ii) is positioned for expression in the cell, under conditions for expressing the isolated DNA, and (b) isolating the

recombinant DAF-16 polypeptide.

A substantially pure antibody, such as a monoclonal or polyclonal antibody, that specifically recognizes and binds a DAF-16 polypeptide is also included in the invention.

5           The invention also features a method of detecting a gene, or a portion of a gene, that is found in a human cell and has sequence identity to the *daf-16* sequence of Figs. 13A or 13B. In this method, isolated DNA encoding a DAF-16 polypeptide, a portion of such DNA that is greater than about 12 residues in length, or a degenerate oligonucleotide corresponding to SEQ ID NO: 54, 55, 56, or 57, is  
10           contacted with a preparation of DNA from the human cell under hybridization conditions that provide detection of DNA sequences having about 70% or greater nucleic acid sequence identity to the *daf-16* sequence of Figs. 13A or 13B. This method can also include a step of testing the gene, or portion of the gene, for the ability to functionally complement a *C. elegans daf-16* mutant.

15           Another method included in the invention is a method of isolating a gene, or a portion of a gene, that is found in a human cell and has at least 90% nucleic acid sequence identity to a sequence encoding SEQ ID NO: 54, 55, 56, or 57. This method involves (a) amplifying by PCR the human gene, or portion thereof, using oligonucleotide primers that (i) are each greater than about 12  
20           residues in length, and (ii) each have regions of complementarity to opposite DNA strands in a region of the nucleotide sequence of Figs. 13A or 13B, and (b) isolating the human gene, or portion thereof. This method can also include a step of testing the gene, or portion thereof, for the ability to functionally complement a *C. elegans daf-16* mutant.

25           In another aspect, the invention features a method of determining

whether a human gene is involved in an impaired glucose tolerance condition (for example, a condition involving atherosclerosis) or obesity. This method involves (a) providing a nematode having a mutation in a *daf* or *age* gene, and (b) expressing in the nematode the human gene, which is operatively linked to a nematode gene promoter. Complementation of the *daf* or *age* mutation in the nematode is indicative of a human gene that is involved in an impaired glucose tolerance condition or obesity. In preferred embodiments, the nematode gene promoter is selected from the group consisting of *daf-1*, *daf-3*, *daf-4*, *daf-2*, *age-1*, and *akt* gene promoters. In other preferred embodiments, the *daf* mutation is selected from the group consisting of *daf-2*, *daf-3*, *daf-1*, *daf-4*, *daf-7*, *daf-8*, *daf-11*, *daf-12*, *daf-14*, and *daf-16* mutations. In yet another preferred embodiment, the mutation can also be found in the *age-1* gene.

In further aspects, the invention features methods for diagnosing an impaired glucose tolerance condition (for example, Type II diabetes or a condition involving atherosclerosis), or a propensity for such a condition, in a patient. One such method includes analyzing the DNA of the patient to determine whether the DNA contains a mutation in a *daf* gene. Identification of such a mutation indicates that the patient has an impaired glucose tolerance condition or a propensity for such a condition. The analysis in this method can be carried out, for example, by nucleotide sequencing or RFLP analysis. The analysis can also include amplifying (for example, by PCR or reverse transcriptase PCR) the gene (for example, a human gene), or a fragment thereof, using primers, and analyzing the amplified gene, or a fragment thereof, for the presence of the mutation. In preferred embodiments, the *daf* gene analyzed in this method is, for example, a *daf-1*, *daf-2*, *daf-3*, *daf-4*, *daf-7*, *daf-8*, *daf-11*, *daf-12*, *daf-14*, *daf-16*, *akt-1*, *akt-2*, *pdk-1*, or *daf-*

18(PTEN) coding sequence, or the *daf* gene is FKHR, FKHRL1, or AFX.

Another method for diagnosing an impaired glucose tolerance condition, such as Type II diabetes, or a propensity for such a condition, in a patient, includes analyzing the DNA of the patient to determine whether the DNA contains a  
5 mutation in an *age* gene. Identification of such a mutation indicates that the patient has an impaired glucose tolerance condition or a propensity for such a condition. The analysis in this method can be carried out, for example, by nucleotide sequencing or RFLP analysis. The analysis can also include amplifying (for example, by PCR or reverse transcriptase PCR) the gene (for example, a  
10 human gene), or a fragment thereof, using primers and analyzing the amplified gene, or fragment thereof, for the presence of the mutation. In a preferred embodiment, the *age* gene is an *age-1* coding sequence.

Yet another method for diagnosing an impaired glucose tolerance condition, such as Type II diabetes or a condition that involves atherosclerosis, or  
15 a propensity for such a condition, in a patient, includes analyzing the DNA of the patient to determine whether the DNA contains a mutation in an *akt* gene. Identification of such a mutation indicates that the patient has an impaired glucose tolerance condition (for example, Type II diabetes) or a propensity for such a condition (for example, a pre-diabetic condition). The analysis in this method can  
20 be carried out, for example, by nucleotide sequencing or RFLP analysis. The analysis can also include amplifying (for example, by PCR or reverse transcriptase PCR) the gene (for example, a human gene), or a fragment thereof, using primers and analyzing the amplified gene, or fragment thereof, for the presence of the mutation.

25 The invention also includes kits for use in the diagnosis of an impaired

glucose tolerance condition, or a propensity for such a condition, in a patient. One such kit includes a PCR primer complementary to a *daf* nucleic acid sequence and instructions for diagnosing an impaired glucose tolerance condition or a propensity for such a condition. Another kit includes a PCR primer complementary to an *age* nucleic acid sequence and instructions for diagnosing an impaired glucose tolerance condition or a propensity for such a condition. Yet another kit includes a PCR primer complementary to an *akt* nucleic acid sequence and instructions for diagnosing an impaired glucose tolerance condition or a propensity for such a condition.

In another aspect, the invention features methods for ameliorating or delaying the onset of an impaired glucose tolerance condition (for example, Type II diabetes) in a patient. In one such method a therapeutically effective amount of a DAF polypeptide (for example, the human or nematode DAF-7 polypeptide) is administered to the patient. In another method, which can be used, for example, in the case of a condition involving atherosclerosis, a therapeutically effective amount of a compound that is capable of inhibiting the activity of a DAF-16 or DAF-3 polypeptide is administered to the patient. In yet another method, a therapeutically effective amount of a compound that activates a DAF-1, DAF-4, DAF-8, DAF-11, or DAF-14 polypeptide is administered to the patient.

Another aspect of the invention provides methods for ameliorating or preventing obesity (for example, obesity associated with Type II diabetes) in a patient. One such method involves administering to the patient a therapeutically effective amount of a DAF polypeptide, such as a human or nematode DAF-7 polypeptide. Another such method involves administering to the patient a therapeutically effective amount of a compound that is capable of inhibiting the

activity of a DAF-16, DAF-3, or DAF-18 (PTEN) polypeptide.

Yet another aspect of the invention features a transgenic, non-human animal, such as a mouse or a nematode, whose germ cells and somatic cells contain a transgene coding for a mutant DAF polypeptide, for example, a mutant  
5 DAF polypeptide that is derived from a human. In preferred embodiments, the mutant DAF polypeptide is a DAF-1, DAF-2, DAF-3, DAF-4, DAF-7, DAF-8, DAF-11, DAF-12, DAF-14, DAF-16, or DAF-18 (PTEN) polypeptide. In another preferred embodiment, the transgene includes a knockout mutation.

In a related aspect, the invention features a transgenic, non-human  
10 animal, such as a mouse or a nematode, whose germ cells and somatic cells contain a transgene coding for a mutant AGE polypeptide, for example, a mutant AGE polypeptide derived from a human. In a preferred embodiment, the mutant AGE polypeptide is an AGE-1 polypeptide. In another preferred embodiment, the transgene includes a knockout mutation.

In yet another aspect, the invention features a transgenic, non-human  
15 animal, such as a mouse or a nematode, whose germ cells and somatic cells contain a transgene coding for a mutant AKT polypeptide, for example, a mutant AKT polypeptide derived from a human. In a preferred embodiment, the transgene includes a knockout mutation.

In related aspects, the invention features cells (for example, cells  
20 isolated from a mammal, such as mouse, human, or nematode cells) isolated from the transgenic animals described above.

The invention also includes methods for producing transgenic, non-human animals. For example, the invention includes a method for producing a  
25 transgenic, non-human animal that lacks an endogenous *daf* gene and is capable of

expressing a human DAF polypeptide. This method involves (a) providing a transgenic, non-human animal whose germ cells and somatic cells contain a mutation in a *daf* gene, and (b) introducing a transgene that (i) encodes a human DAF polypeptide, and (ii) is capable of expressing the human polypeptide, into an embryonal cell of the non-human animal.

Another method included in the invention can be used for producing a transgenic, non-human animal that lacks an endogenous *age* gene and is capable of expressing a human AGE polypeptide. This method involves (a) providing a transgenic, non-human animal whose germ cells and somatic cells contain a mutation in an *age* gene, and (b) introducing a transgene that (i) encodes a human AGE polypeptide, and (ii) is capable of expressing the human polypeptide, into an embryonal cell of the non-human animal.

Similarly, the invention includes a method for producing a transgenic, non-human animal that lacks an endogenous *akt* gene and is capable of expressing of expressing a human AKT polypeptide. This method involves (a) providing a transgenic, non-human animal whose germ cells and somatic cells contain a mutation in an *akt* gene, and (b) introducing a transgene that (i) encodes a human AKT polypeptide, and (ii) is capable of expressing the human polypeptide, into an embryonal cell of the non-human animal.

Another aspect of the invention features a method of screening for a compound that increases the activity of a DAF polypeptide. This method includes (a) exposing a non-human transgenic animal whose germ cells and somatic cells contain a transgene coding for a mutant DAF polypeptide to a candidate compound, and (b) determining the activity of the DAF polypeptide in the transgenic animal. An increase in DAF polypeptide activity, as compared to

untreated controls, is indicative of a compound that is capable of increasing DAF polypeptide activity. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition or obesity.

In a related aspect, the invention features a method of screening for a compound that decreases the activity of a DAF polypeptide. This method includes (a) exposing a non-human transgenic animal whose germ cells and somatic cells contain a transgene coding for a mutant DAF polypeptide to a candidate compound, and (b) determining the activity of the DAF polypeptide in the transgenic animal. A decrease in DAF polypeptide activity, as compared to untreated controls, is indicative of a compound that is capable of decreasing DAF polypeptide activity. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition, obesity, or atherosclerosis. In other preferred embodiments, the compound decreases the activity of DAF-3 or DAF-16.

In another aspect, the invention features a method of screening for a compound that increases the activity of an AGE polypeptide. This method includes (a) exposing a non-human transgenic animal whose germ cells and somatic cells contain a transgene coding for a mutant AGE polypeptide to a candidate compound, and (b) determining the activity of the AGE polypeptide in the transgenic animal. An increase in AGE polypeptide activity, as compared to untreated controls, is indicative of a compound that is capable of increasing AGE polypeptide activity. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition, obesity, or atherosclerosis.

In a related aspect, the invention features a method of screening for a compound that decreases the activity of a AGE polypeptide. This method includes

(a) exposing a non-human, transgenic animal whose germ cells and somatic cells contain a transgene coding for a mutant AGE polypeptide to a candidate compound, and (b) determining the activity of the AGE polypeptide in the transgenic animal. A decrease in AGE polypeptide activity, as compared to  
5 untreated controls, is indicative of a compound that is capable of decreasing AGE polypeptide activity. In preferred embodiments, the compound can be used to treat or delay aging. In another preferred embodiment, the AGE polypeptide is AGE-1.

In another aspect, the invention features a method of screening for a compound that increases the activity of an AKT polypeptide. This method  
10 includes (a) exposing a transgenic, non-human animal whose germ cells and somatic cells contain a transgene coding for a mutant AKT polypeptide to a candidate compound, and (b) determining the activity of the AKT polypeptide in the transgenic animal. An increase in AKT polypeptide activity, as compared to untreated controls, is indicative of a compound that is capable of increasing AKT  
15 polypeptide activity. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition, obesity, or atherosclerosis.

In a related aspect, the invention features a method of screening for a compound that decreases the activity of a AKT polypeptide. This method includes  
20 (a) exposing a transgenic, non-human animal whose germ cells and somatic cells contain a transgene coding for a mutant AKT polypeptide to a candidate compound, and (b) determining the activity of the AKT polypeptide in the transgenic animal. A decrease in AKT polypeptide activity, as compared to untreated controls, is indicative of a compound that is capable of decreasing AKT polypeptide activity. In preferred embodiments, the compound can be used to treat  
25 or delay aging.

Also included in the invention is a method of screening for a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition. This method involves (a) exposing a transgenic, non-human animal whose germ cells and somatic cells contain a transgene coding for a mutant DAF, AGE, or AKT polypeptide to a candidate compound, and (b) monitoring the blood glucose level of the animal. A compound that promotes maintenance of a physiologically acceptable level of blood glucose in the animal, as compared to untreated controls, is indicative of a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition. In a preferred embodiment, the compound can be used to treat Type II diabetes.

Another method of screening for a compound that is capable of ameliorating or delaying obesity is also included in the invention. This method involves (a) exposing a transgenic, non-human animal whose germ cells and somatic cells contain a transgene coding for a mutant DAF, AGE, or AKT polypeptide to a candidate compound, and (b) monitoring the adipose tissue of the animal. A compound that promotes maintenance of a physiologically acceptable level of adipose tissue in the animal, as compared to untreated controls, is indicative of a compound that is capable of ameliorating or delaying obesity.

A related method of the invention can be used for screening for a compound that is capable of ameliorating or delaying atherosclerosis. This method involves (a) exposing a transgenic, non-human animal whose germ cells and somatic cells contain a transgene coding for a mutant DAF, AGE, or AKT polypeptide to a candidate compound, and (b) monitoring the adipose tissue of the animal. A compound that promotes maintenance of a physiologically acceptable level of adipose tissue in the animal, as compared to untreated controls, is

indicative of a compound that is capable of ameliorating or delaying atherosclerosis.

In another aspect, the invention includes a method for identifying a modulatory compound that is capable of decreasing the expression of a *daf* gene.

- 5 This method involves (a) providing a cell expressing the *daf* gene, and (b) contacting the cell with a candidate compound. A decrease in *daf* expression following contact with the candidate compound identifies a modulatory compound. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition or obesity. In other preferred embodiments, the compound is
- 10 capable of decreasing the expression of DAF-3 or DAF-16. This method can be carried out in an animal, such as a nematode.

- In a related aspect, the invention includes a method for the identification of a modulatory compound that is capable of increasing the expression of a *daf* gene. This method involves (a) providing a cell expressing the *daf* gene, and (b)
- 15 contacting the cell with a candidate compound. An increase in *daf* expression following contact with the candidate compound identifies a modulatory compound. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition or obesity. In other preferred embodiments, the compound is capable of increasing expression of DAF-1, DAF-2, DAF-4, DAF-7, DAF-8,
- 20 DAF-11, or DAF-14. This method can be carried out in an animal, such as a nematode.

- In another aspect, the invention includes a method for the identification of a modulatory compound that is capable of increasing the expression of an *age-1* gene. This method involves (a) providing a cell expressing the *age-1* gene, and (b)
- 25 contacting the cell with a candidate compound. An increase in *age-1* expression

following contact with the candidate compound identifies a modulatory compound. In preferred embodiments, the compound is capable of treating an impaired glucose tolerance condition or obesity. This method can be carried out in an animal, such as a nematode.

5           In another aspect, the invention provides a method for identification of a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition. This method involves (a) providing a dauer larvae including a mutation in a *daf* gene, and (b) contacting the dauer larvae with a compound. Release from the dauer larval state is an indication that the compound is capable of  
10   ameliorating or delaying an impaired glucose tolerance condition. In a preferred embodiment, the dauer larvae carries a *daf-2* mutation. In another preferred embodiment, the dauer larvae is from *C. elegans*. In yet another embodiment, the impaired glucose tolerance condition involves obesity or atherosclerosis.

          In a related aspect, the invention provides a method for identification of  
15   a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition. This method involves (a) providing a dauer larvae including a mutation in an *age-1* gene, and (b) contacting the dauer larvae with a compound. Release from the dauer larval state is an indication that the compound is capable of  
20   ameliorating or delaying an impaired glucose tolerance condition. In a preferred embodiment, the dauer larvae carries an *age-1* mutation. In another preferred embodiment, the dauer larvae is from *C. elegans*. In yet another preferred embodiment, the impaired glucose tolerance condition involves obesity or atherosclerosis.

          In another related aspect, the invention provides a method for the  
25   identification of a compound that is capable of ameliorating or delaying an

impaired glucose tolerance condition. This method involves (a) providing a dauer larvae including a mutation in an *akt* gene, and (b) contacting the dauer larvae with a compound. Release from the dauer larval state is an indication that the compound is capable of ameliorating or delaying an impaired glucose tolerance condition. In a preferred embodiment, the dauer larvae is from *C. elegans*. In another preferred embodiment, the impaired glucose tolerance condition involves obesity or atherosclerosis.

In another aspect, the invention provides a method for the identification of a compound for ameliorating or delaying an impaired glucose tolerance condition. This method involves (a) combining PIP3 and an AKT polypeptide in the presence and absence of a compound under conditions that allow PIP3:AKT complex formation, (b) identifying a compound that is capable of decreasing the formation of the PIP3:AKT complex, and (c) determining whether the compound identified in step (b) is capable of increasing AKT activity. An increase in AKT kinase activity is taken as an indication of a compound useful for ameliorating or delaying an impaired glucose tolerance condition.

In yet another aspect, the invention provides a method for the identification of a compound for ameliorating or delaying an impaired glucose tolerance condition. This method involves (a) providing a *daf-7*, *daf-3* mutant nematode, (b) expressing in the cells of the nematode a mammalian DAF-3 polypeptide, whereby the nematode forms a dauer larva, and (c) contacting the dauer larva with a compound. A release from the dauer larval state is an indication that the compound is capable of ameliorating or delaying the glucose intolerance condition.

In a further aspect, the invention features a method for the identification

of a compound for ameliorating or delaying an impaired glucose tolerance condition. This method involves (a) providing a *daf-2*, *daf-16* mutant nematode, (b) expressing in the cells of the nematode a mammalian DAF-16 polypeptide, whereby the nematode forms a dauer larva, and (c) contacting the dauer larva with a compound. A release from the dauer larval state is an indication that the compound is capable of ameliorating or delaying the glucose intolerance condition.

In yet another aspect, the invention features insulin-like molecules and their use as diagnostic and therapeutic reagents.

As used herein, by a “DAF” polypeptide is meant a polypeptide that functionally complements a *C. elegans daf* mutation and/or that has at least 60%, preferably 75%, and more preferably 90% amino acid sequence identity to a 100 amino acid region (and preferably a conserved domain) of a *C. elegans* DAF polypeptide. Complementation may be assayed in an organism (for example, in a nematode) or in a cell culture system. Complementation may be partial or complete, but must provide a detectable increase in function (as described herein). DAF polypeptides are encoded by “DAF” genes or nucleic acid sequences.

By an “AGE” polypeptide is meant a polypeptide that functionally complements a *C. elegans age* mutation and/or that has at least 60%, preferably 75%, and more preferably 90% amino acid sequence identity to a 100 amino acid region (and preferably a conserved domain) of a *C. elegans* AGE polypeptide. Complementation may be assayed in an organism (for example, in a nematode) or in a cell culture system. Complementation may be partial or complete, but must provide a detectable increase in a known AGE function. AGE polypeptides are encoded by “AGE” genes or nucleic acid sequences.

As used herein, by an "AKT" polypeptide is meant a polypeptide that functionally complements a *C. elegans akt* mutation and/or that possess at least 64% amino acid sequence identity to SEQ ID NO: 60, at least 71% amino acid sequence identity to SEQ ID NO: 61, at least 79% amino acid sequence identity to SEQ ID NO: 62, at least 63% amino acid sequence identity to SEQ ID NO: 63, at least 48% amino acid sequence identity to SEQ ID NO: 64, at least 70% amino acid sequence identity to SEQ ID NO: 65, at least 64% amino acid sequence identity to SEQ ID NO: 66, at least 67% amino acid sequence identity to SEQ ID NO: 67, or a combination thereof. Complementation may be assayed in an organism (for example, in a nematode) or in a cell culture system. Complementation may be partial or complete, but must provide a detectable increase in a known AKT function. AKT polypeptides are encoded by "AKT" genes or nucleic acid sequences.

By a "DAF-2 polypeptide" is meant a polypeptide that complements (as defined above) a *C. elegans daf-2* mutation and/or that possesses at least 61% amino acid sequence identity to SEQ ID NO: 33, at least 31% amino acid sequence identity to SEQ ID NO: 34, at least 43% amino acid sequence identity to SEQ ID NO: 79, at least 35% amino acid sequence identity to SEQ ID NO: 80, at least 35% amino acid sequence identity to SEQ ID NO: 81, at least 48% amino acid sequence identity to SEQ ID NO: 82, at least 43% amino acid sequence identity to SEQ ID NO: 83, at least 40% amino acid sequence identity to SEQ ID NO: 84, or a combination thereof. Preferably, a DAF-2 polypeptide includes an aspartic acid, a proline, a proline, a serine, an alanine, an aspartic acid, a cysteine, or a proline at amino acid positions corresponding to *C. elegans* DAF-2 amino acids 1252, 1312, 1343, 347, 451, 458, 526, 279, and 348 respectively, or a combination thereof.

By a "DAF-3 polypeptide" is meant a polypeptide that complements (as defined above) a *C. elegans daf-3* mutation and/or that possesses at least 60% amino acid sequence identity to SEQ ID NO: 35, at least 38% amino acid sequence identity to SEQ ID NO: 36, at least 47% amino acid sequence identity to SEQ ID NO: 85, or a combination thereof. Preferably, a DAF-3 polypeptide includes a proline or a glycine at amino acid positions corresponding to *C. elegans daf-3* amino acids at positions 200 (proline) and/or 620 (glycine) in Fig. 12A, respectively, or a combination thereof. For example, the polypeptide may include a proline in the motif GRKGFPHV (SEQ ID NO: 200) or a glycine in the motif RXXIXXG (where X is any amino acid) (SEQ ID NO: 201).

By a "DAF-16 polypeptide" is meant a polypeptide that complements (as defined above) a *C. elegans daf-16* mutation and/or that possesses at least 71% amino acid sequence identity to SEQ ID NO: 54, at least 35% amino acid sequence identity to SEQ ID NO: 55, at least 65% amino acid sequence identity to SEQ ID NO: 56, at least 53% amino acid sequence identity to SEQ ID NO: 57, or a combination thereof. In addition, a DAF-16 polypeptide preferably includes a serine residue in the conserved motif WKNSIRH (SEQ ID NO: 59).

By a "DAF-7 polypeptide" is meant a polypeptide that complements (as defined above) a *C. elegans daf-7* mutation and/or that possesses at least 29% amino acid sequence identity to SEQ ID NO: 26, at least 66% amino acid sequence identity to SEQ ID NO: 27, at least 45% amino acid sequence identity to SEQ ID NO: 28, at least 33% amino acid sequence identity to SEQ ID NO: 29, at least 56% amino acid sequence identity to SEQ ID NO: 30, at least 75% sequence identity to SEQ ID No: 51, or a combination thereof. Preferably, a DAF-7 polypeptide includes a proline or a glycine at amino acid positions corresponding to *C. elegans*

*daf-7* amino acids 271 and 280, respectively, or a combination thereof.

By a “DAF-8 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-8* mutation and/or that possesses at least 46% amino acid sequence identity to SEQ ID NO: 23, at least 45% amino acid sequence identity to SEQ ID NO: 24, at least 36% amino acid sequence identity to SEQ ID NO: 25, or a combination thereof.

By an “AGE-1 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans age-1* mutation (previously known as a *daf-23* mutation) and/or that possesses at least 40% amino acid sequence identity to SEQ ID NO: 17, at least 45% amino acid sequence identity to SEQ ID NO: 18, at least 30% amino acid sequence identity to SEQ ID NO: 19, at least 24% amino acid sequence identity to SEQ ID NO: 38, or a combination thereof. Preferably, an AGE-1 polypeptide includes an alanine at amino acid positions corresponding to *C. elegans age-1* amino acids 845.

By a “DAF-1 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-1* mutation and/or that possesses at least 45% amino acid sequence identity to SEQ ID NO: 13, at least 35% amino acid sequence identity to SEQ ID NO: 14, at least 65% amino acid sequence identity to SEQ ID NO: 15, at least 25% amino acid sequence identity to SEQ ID NO: 16, or a combination thereof. Preferably, a DAF-1 polypeptide includes a proline at the amino acid position corresponding to *C. elegans* DAF-1 amino acid 546.

By a “DAF-4 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-4* mutation and/or that possesses at least 45% amino acid sequence identity to SEQ ID NO: 20, at least 40% amino acid sequence identity to SEQ ID NO: 21, at least 44% amino acid sequence identity to SEQ ID

NO: 22, or a combination thereof.

By a “DAF-11 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-11* mutation and/or that possesses at least 40% amino acid sequence identity to SEQ ID NO: 75, at least 43% amino acid sequence identity to SEQ ID NO: 76, at least 36% amino acid sequence identity to SEQ ID NO: 77, at least 65% amino acid sequence identity to SEQ ID NO: 78, or a combination thereof.

By a “DAF-12 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-12* mutation and/or that possesses at least 42% amino acid sequence identity to SEQ ID NO: 72, at least 58% amino acid sequence identity to SEQ ID NO: 73, at least 34% amino acid sequence identity to SEQ ID NO: 74, or a combination thereof.

By a “DAF-14 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-14* mutation and/or that possesses at least 48% amino acid sequence identity to SEQ ID NO: 68, at least 37% amino acid sequence identity to SEQ ID NO: 69, at least 48% amino acid sequence identity to SEQ ID NO: 70, at least 37% amino acid sequence identity to SEQ ID NO: 71, or a combination thereof.

By a “PTEN” polypeptide is meant a PTEN lipid phosphatase from any animal. Preferably, this animal is a mammal and, most preferably, a human. This polypeptide is also referred to as MMAC1 and TEP1.

By “insulin receptor activity” is meant any activity exhibited by an insulin receptor and measured by either (i) activation of insulin receptor substrate-1 (IRS-1) phosphorylation and recruitment of PI-3 kinase, (ii) activation of glucose transporter (Glut 4) fusion with a cellular membrane and concomitant glucose

uptake, or (iii) activation of glycogen and/or fat synthesis and concomitant inhibition of gluconeogenesis or lipolysis or both.

By “insulin receptor related activity” is meant any activity not directly attributable to the insulin receptor but that is measured by an activation of IRS-1 phosphorylation and recruitment of PI3-kinase.

By “IGF-1 receptor activity” is meant any activity exhibited by an insulin-like growth factor-1 receptor and measured by (i) activation of IRS-1 phosphorylation and recruitment of PI-3 kinase, (ii) activation of cell division in NIH3T3 cells (e.g., as described in Gronborg et al., J. Biol. Chem. 268: 23435-23440, 1993), or (iii) activation of bone growth in, for example, the mouse model.

By “SMAD protein” is meant a protein that is capable of coupling to TGF- $\beta$  type ser/thr receptors. Smad proteins typically contain a smad conserved motif as described by Derynk et al. (*Cell* 87: 173, 1996). Exemplary smad proteins include, without limitation, DAF-3, MADR-2, MAD, DPC-4, and Sma-2.

By “AKT activity” is meant any activity exhibited by an AKT polypeptide and measured by phosphatidylinositol-regulated increases in serine phosphorylation of GSK-3, DAF-16, AFX, FKHR, or FKHL1, or activation of non-dauer growth in *C. elegans akt* mutants.

By “impaired glucose tolerance condition” is meant any condition in which blood sugar levels are inappropriately elevated or lack normal metabolic regulation. Examples of such conditions include, without limitation, Type I diabetes, Type II diabetes, and gestational diabetes, and may be associated with obesity and atherosclerosis.

By "protein" or "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or

phosphorylation).

By "substantially pure" is meant a preparation which is at least 60% by weight (dry weight) the compound of interest, e.g., any of the polypeptides of the invention such as the DAF-2, DAF-3, or DAF-16 polypeptides or DAF-2, DAF-3, or DAF-16-specific antibodies. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

By "isolated DNA" is meant DNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By a "substantially identical" polypeptide sequence is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the function of the polypeptide (assayed, e.g., as described herein).

Preferably, such a sequence is at least 75%, more preferably 85%, and most preferably 95% identical at the amino acid level to the sequence used for comparison.

Homology is typically measured using sequence analysis software (e.g.,  
5 Sequence Analysis Software Package of the Genetics Computer Group, University  
of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705  
or BLAST software available from the National Library of Medicine). Examples  
of useful software include the programs, Pileup and PrettyBox. Such software  
matches similar sequences by assigning degrees of homology to various  
10 substitutions, deletions, substitutions, and other modifications. Conservative  
substitutions typically include substitutions within the following groups: glycine,  
alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine,  
glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By a "substantially identical" nucleic acid is meant a nucleic acid  
15 sequence which encodes a polypeptide differing only by conservative amino acid  
substitutions, for example, substitution of one amino acid for another of the same  
class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-  
conservative substitutions, deletions, or insertions located at positions of the amino  
acid sequence which do not destroy the function of the polypeptide (assayed, e.g.,  
20 as described herein). Preferably, the encoded sequence is at least 75%, more  
preferably 85%, and most preferably 95% identical at the amino acid level to the  
sequence of comparison. If nucleic acid sequences are compared a "substantially  
identical" nucleic acid sequence is one which is at least 85%, more preferably  
90%, and most preferably 95% identical to the sequence of comparison. The  
25 length of nucleic acid sequence comparison will generally be at least 50

5 nucleotides, preferably at least 60 nucleotides, more preferably at least 75  
nucleotides, and most preferably 110 nucleotides. Again, homology is typically  
measured using sequence analysis software (e.g., Sequence Analysis Software  
Package of the Genetics Computer Group, University of Wisconsin Biotechnology  
Center, 1710 University Avenue, Madison, WI 53705).

10 By "positioned for expression" is meant that the DNA molecule is  
positioned adjacent to a DNA sequence which directs transcription and translation  
of the sequence (i.e., facilitates the production of any of the polypeptides disclosed  
herein including, but not limited to, DAF-2, DAF-3, and DAF-16 and any human  
homolog thereof).

By "purified antibody" is meant antibody which is at least 60%, by  
weight, free from the proteins and naturally-occurring organic molecules with  
which it is naturally associated. Preferably, the preparation is at least 75%, more  
preferably at least 90%, and most preferably at least 99%, by weight, antibody.

15 By "specifically binds" is meant an antibody which recognizes and binds  
a polypeptide of the invention (e.g., DAF-2, DAF-3, and DAF-16) but which does  
not substantially recognize and bind other molecules in a sample (e.g., a biological  
sample) which naturally includes a polypeptide of the invention. An antibody  
which "specifically binds" such a polypeptide is sufficient to detect protein product  
20 in such a biological sample using one or more of the standard immunological  
techniques available to those in the art (for example, Western blotting or  
immunoprecipitation).

By "immunological methods" is meant any assay involving antibody-  
based detection techniques including, without limitation, Western blotting,  
25 immunoprecipitation, and direct and competitive ELISA and RIA techniques.

By "means for detecting" is meant any one or a series of components that sufficiently indicate a detection event of interest. Such means involve at least one label that may be assayed or observed, including, without limitation, radioactive, fluorescent, and chemiluminescent labels.

5 By "hybridization techniques" is meant any detection assay involving specific interactions (based on complementarity) between nucleic acid strands, including DNA-DNA, RNA-RNA, and DNA-RNA interactions. Such hybridization techniques may, if desired, include a PCR amplification step.

By a "modulatory compound", as used herein, is meant any compound  
10 capable of either decreasing DAF-3, DAF-16, or DAF-18 (PTEN) expression (i.e., at the level of transcription, translation, or post-translation) or decreasing DAF-3, DAF-16, or DAF-18 (PTEN) protein levels or activity. Also included are compounds capable of either increasing DAF-1, DAF-2, DAF-4, DAF-8, DAF-7, DAF-11, DAF-14, AGE-1, AKT, or PDK1 expression (i.e., at the level of  
15 transcription, translation, or post-translation) or increasing DAF-1, DAF-2, DAF-4, DAF-8, DAF-7, DAF-11, DAF-14, AGE-1, AKT, or PDK-1 protein levels or their corresponding activities.

By "complementation" is meant an improvement of a genetic defect or mutation. In one example, complementation of a genetic defect in a *daf*, *age*, or  
20 *akt* gene can be carried out by providing the wild-type *daf*, *age*, or *akt* genes, respectively. Complementation is generally accomplished by expressing the wild-type version of the protein in a host cell or animal bearing a mutant or inactive version of the gene.

Other features and advantages of the invention will be apparent from the  
25 following detailed description thereof, and from the claims.

### Detailed Description

The drawings will first be described.

#### Drawings

5           **Fig. 1** shows the genetic and physical map of *C. elegans daf-2*. The top panel shows the genetic map of *daf-2*. *daf-2* maps on the left arm of chromosome III 11.4 map units to the right of *dpy-1* and 1.6 map units to the left of *ben-1* (ACeDB). The middle panel shows the physical map of *daf-2*. *daf-2* maps between *mgP34* and *mgP44* in a region not covered by cosmid clones but covered  
10 by YAC Y53G8. Cosmids from the approximate *daf-2* genetic location detect RFLPs between *C. elegans* strains Bristol N2 and Bergerac RC301. *mgP31* on cosmid T21A6 is a HindIII RFLP: 5.3 kb in Bristol, 4.5 kb in RC301. *mgP33* on cosmid T02B2 is a HindIII RFLP: 9 kb in Bristol, 8 kb in RC301. *mgP34* on cosmid R10F2 is an EcoRI RFLP: 4.1 and 2.8 kb in Bristol, 3.6 kb in RC301.  
15 *mgP44* on cosmid R07G11 is a complex EcoRI RFLP: 2.9 kb, 2.4 kb, 1.9 kb and 1.7kb in Bristol; 3.6kb, 2.5kb and 1.6kb in RC301. *mgP35* on cosmid T10D5 is a StyI RFLP: 5.4 kb in Bristol, 5.8 kb in RC301. *mgP32* on cosmid C42B8 is a StyI RFLP: 2.8 kb in Bristol; 2.9kb in RC301. *mgP48* detected with *daf-2* probe (nt 1277-2126 and 3747-4650) is a HindIII RFLP: 4.3kb and 7kb in Bristol and 4.1kb  
20 and 6.2kb in RC301. Thirty-one out of thirty-three Dpy-non-Daf recombinants carry the RC301 allele of *mgP34* whereas all thirty-three recombinants in this interval carry the RC301 allele of *mgP44*, mapping *daf-2* 0.69 map units to the right of *mgP34* and to the left of *mgP44*. Fourteen out of twenty-four Ben-non-Daf recombinants carry the RC301 *mgP44* allele whereas all of these  
25 recombinants carry the RC301 allele of *mgP34*, mapping *daf-2* 0.66 map units to

the left of *mgP44*.

Y53G8 YAC DNA was isolated from CHEF gels as described in Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y., 1990), labeled , and shown to hybridize to multiple restriction

5 fragments from cosmids bearing *mgP34* and *mgP44*. A probe from the insulin receptor homolog on Y53G8 detects the *mgP48* RFLP between N2 and RC301. All thirty-three Dpy-non-Daf and all twenty-four Ben-non-Daf recombinants described above carry the RC301 allele of *mgP48*, indicating that *daf-2* could not be separated from this insulin receptor gene by these fifty-seven recombination  
10 events in a thirteen map unit interval.

The bottom panel shows the structure of *daf-2* cDNA. The *daf-2* cDNA was amplified from a cDNA library constructed according to standard methods by PCR using internal primers derived from the genomic shotgun sequences, vector sequence primers (for 3' end) and an SL1 transspliced leader PCR primer  
15 (M. Krause, In: *Methods Cell Biol.*, vol. 48, pp. 483-512, H. F. Epstein and D. C. Shakes, eds., Academic Press, San Diego, CA, 1995). To isolate a cDNA, pooled plasmid DNA from 106 clones of a 107 clone complexity cDNA library was used as a PCR template. To obtain a *daf-2* cDNA 3' end, *daf-2* internal primer CGCTACGGCAAAAAAGTGAA (SEQ ID NO: 1) in the kinase domain and a  
20 cloning vector primer CGATGATGAAGATACCCC (SEQ ID NO: 2) were used in a nested PCR reaction with adjacent internal primers. For the cDNA fragment from the ligand-binding domain to the kinase domain, PCR was carried out with TGATGCGAACGGCGATCGAT (SEQ ID NO: 3) and ACGCTGGATCATCTACATTA (SEQ ID NO: 4) primers. For the *daf-2* 5' end,  
25 SL1 primer GGTTTAATTACCCAAGTTTGAG (SEQ ID NO: 5) and one

internal *daf-2* primer GCTCACGGGTACACAACGA (SEQ ID NO: 6) were used in a nested PCR reaction with adjacent internal primers. Using PCR to amplify genomic DNA from a set of 20 *daf-2* mutants, we searched for *daf-2* mutations in a 0.8 kb region of the ligand binding domain and in a 0.9 kb region of the kinase domain. For sequencing the ligand-binding domain PCR primers TGATGCGAACGGCGATCGAT (SEQ ID NO: 7) and TGAGGGCCAACTAAAGAAGAC (SEQ ID NO: 8) were used. In the kinase domain primers CGCTACGGCAAAAAAGTGAA (SEQ ID NO: 9) and GACGATCCCGAGGTGAGTAT (SEQ ID NO: 10) were used. The presence of an SL1 spliced leader sequence indicates a full length *daf-2* cDNA. The predicted ORF is shown as a box; 5' and 3' UTRs are shown as thick bars. The predicted DAF-2 initiator methionine at base 486 is preceded by an in frame stop codon 63 bases upstream. The predicted DAF-2 stop codon is found at base 5658. No consensus polyadenylation signal was found in the cDNA nor in genomic shotgun sequence #00678, which extends 302 bp further downstream. The initial insulin receptor homolog shotgun sequences are shown as thin bars above the box.

Introns were detected by a combination of *in silico* genomic and cDNA sequence comparison, and by comparison of PCR products derived from cDNA and genomic DNA templates. The open triangles over a vertical bar indicate positions of the detected exon/intron boundaries. All the intron donor sites have GT consensus and the acceptor sites have AG consensus (Krause, 1995 *supra*). The triangles without a vertical bar indicate the approximate intron locations determined by comparison of PCR products using genomic DNA or cDNA as a template. Intron lengths were estimated by comparison of the PCR product size using cDNA or genomic DNA templates. Genomic regions corresponding to

some of the introns could not be PCR amplified suggesting that these introns are long. The minimum *daf-2* gene size based on this analysis is 33 kb.

**Fig. 2A** shows the predicted *C. elegans* DAF-2 amino acid sequence. The predicted cysteine-rich region (amino acids 207-372) and tyrosine kinase domain (amino acids 1124-1398) are boxed. The signal peptide (amino acids 1-20), proteolysis site (amino acids 806-809), transmembrane domain (amino acids 1062-1085), and PTB binding motif in the juxtamembrane region (NPEY, amino acids 1103-1106) are underlined. Three DAF-2 tyrosine residues, Y1293, Y1296 and Y1297, in the region corresponding to the insulin receptor kinase Y1158 to Y1163 activation loop are likely to be autophosphorylated, based on the predicted similarity between the DAF-2 and insulin receptor phosphorylation targets (Fig. 2B). Another likely target for DAF-2 autophosphorylation is the Y1106 NPEY motif located in the region corresponding to the insulin receptor juxtamembrane region NPEY motif (at Y972), that has been shown to mediate IRS-1 binding via its PTB domain to the insulin receptor (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). While DAF-2 bears one YXXM motif implicated in coupling to PI 3-kinase, mammalian IRS-1 and *Drosophila* insulin receptor (Fernandez et al., *EMBO J.* 14: 3373-3384, 1995) bear multiple YXXM motifs. Although no p85-like adaptor subunit has yet been detected in the *C. elegans* database, the AGE-1 homology to mammalian p110 suggests the existence of a homologous or analogous adaptor (Morris et al., *Nature* 382: 536-539, 1996). In the DAF-2 C-terminal domain, two other tyrosine residues may be autophosphorylated and bound to particular SH2-containing proteins: Y1678 binding to a PLC-g or SHP-2 homolog, and Y1686, perhaps binding to SEM-5 (Fig. 2A) (Songyang et al., *Cell* 72: 767-778, 1993). While mutations in, for example, ras and MAP kinase have

not been identified in screens for dauer constitutive or dauer defective mutations, these general signaling pathway proteins may couple to DAF-2 as they couple to insulin signaling in vertebrates (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). The predicted phosphotyrosine residues in juxtamembrane region and the kinase domain activation loop are circled. In the extended C-terminal region, predicted phosphotyrosine residues are also circled and SH2-binding sites are underlined (see below).

**Fig. 2B** shows the cDNA encoding the *C. elegans* DAF-2.

**Fig. 2C** shows the amino acid comparison of *C. elegans* DAF-2 to the human insulin receptor and human IGF-I receptor (shown in parenthesis), and to the *Drosophila* insulin receptor homolog, with *daf-2* and human insulin receptor mutations highlighted. Six *daf-2* mutations map in the ligand-binding domain: *sa187* (C347S, TGT to AGT), *e1368* (S451L, TCA to TTA), *e1365* (A458T, GCT to ACT), *sa229* (D526N, GAT to AAT), and two mutations in *mg43* (C279Y, TGT to TAT and P348L, CCC to CTC). Three *daf-2* mutations substitute conserved amino acid residues in the insulin receptor kinase domain: *sa219* (D1252N, GAT to AAT), *e1391* (P1312L, CCC to CTC), and *e1370* (P1343S, CCA to TCA). Darkened residues indicate amino acid identity. Hatched residues indicate amino acid similarity. The percentages under the domains represents the percentage of identity observed between DAF-2 and each receptor. The corresponding BLAST probabilities of DAF-2 random match to each protein is:  $6.4 \times 10^{-157}$  (human insulin receptor),  $2.7 \times 10^{-156}$  (human IGF-I receptor),  $2.1 \times 10^{-153}$  (molluscan InR homolog),  $8.3 \times 10^{-153}$  (mosquito InR homolog),  $1.6 \times 10^{-138}$  (human insulin receptor-related receptor),  $1.7 \times 10^{-122}$  (*Drosophila* InR homolog),  $2.0 \times 10^{-108}$  (Hydra InR homolog). DAF-2 is more distant from the next most closely related

kinase families:  $8.9 \times 10^{-58}$  (v-ros) and  $3.0 \times 10^{-51}$  (trkC neurotrophin receptor).

Conserved cysteine residues in the ligand-binding domain (top) are marked with dots. In the kinase domain, active site residues that mediate insulin receptor kinase specificity are marked with stars. All of these residues are homologous in DAF-2. The mutations found in human patients are indicated at the top of the row, and *daf-2* allele substitutions are indicated below with allele names. The sequence alignments were done with GCG programs, Pileup and Prettybox, and the identities were calculated with the GCG program, Gap.

**Fig. 3** is a photograph showing the metabolic control by *C. elegans* *daf-2* and *daf-7*. The top panel shows low levels of fat accumulation in a wild type L3 animal grown at 25°C that has been stained with Sudan black. Non-starved animals were fixed in 1% paraformaldehyde in PBS, frozen at -70°C, and freeze-thawed three times. Fixed animals were washed three times in PBS, and then incubated overnight in 1X Sudan black according to standard methods. The next panel shows higher levels of fat accumulation in *daf-2(e1370)* grown at the non-permissive temperature of 25°C. These animals accumulate fat in both intestinal and hypodermal cells. *daf-2(e1370)* animals grown at 15°C, the permissive temperature, accumulate low levels of fat, like wild type (data not shown). The next panel shows high fat levels in the intestine and hypodermis of *daf-7(e1372)* animals grown at 25°C. The bottom panel shows high levels of fat in *daf-2(e1370)* animals grown at the permissive temperature until the L4 stage and then shifted to the non-permissive temperature. This shows that *daf-2* regulates metabolism without entry into the dauer stage.

**Fig. 4** is a schematic diagram showing a model of insulin signaling in the *C. elegans* dauer formation pathway. In the absence of dauer pheromone, an

insulin-like ligand activates DAF-2, and DAF-7 TGF- $\beta$ -like signal activates the DAF-1 and DAF-4 receptors. Activated DAF-2 autophosphorylates particular tyrosine residues and recruits signaling molecules, including the PI 3-kinase homolog (a heterodimer of an as yet unidentified p85 homolog and the PI 3-kinase catalytic subunit AGE-1). The AGE-1 PI 3-kinase produces PIP3 second messenger. This second messenger may regulate glucose transport (White and Kahn, 1994 *supra*), metabolic kinase cascades that include AKT and GSK-3 (Hemmings, *Science* 226:1344-1345, 1984; Jonas et al., *Nature*, 385:343-346, 1997), and transcription and translation of metabolic genes (White and Kahn, 1994, *supra*). DAF-16 acts downstream of DAF-2 and AGE-1 in this pathway and is negatively regulated by them (Vowels and Thomas, *Genetics*, 130:105-123, 1992; Gottlieb and Ruvkun, *Genetics*, 137:107-110, 1994). While both the DAF-7/TGF- $\beta$  and DAF-2/insulin signaling pathways converge to control dauer formation, only the DAF-2 pathway controls reproductive phase longevity. This may be due to non-transcriptional outputs of DAF-2 suggested by precedents from insulin receptor signaling. DAF-7 signaling output is predicted to be only transcriptional as described herein.

**Fig. 5A** shows that *C. elegans daf-3* was genetically mapped to a region on the X chromosome between *aex-3* and *unc-1*. Cosmid and plasmid clones from the region were assayed for transformation rescue (Mello et al., *EMBO J* 10: 3959-3970, 1991). Plasmid pRF4 (*rol-6* transformation marker, 100 ng/ml), and cosmids (5-6 ng/ml) were injected into the gonad of *daf-7 (e1372)*; *daf-3 (e1376)* animals. Transgenic animals were scored for dauer formation at 25°C; a dauer (i.e., a return to the *daf-7* phenotype) indicates rescue of *daf-3*; clones that rescue *daf-3* are boxed. B0217 rescues the *daf-3* phenotype; eighteen of nineteen

transgenic lines were rescued (~80% dauers). Examination of sequence provided by the *C. elegans* Sequencing Consortium revealed a Smad homologous gene on B0217. A 13 kb subclone of B0217 containing just the Smad also rescues *daf-3* (see Fig. 3). No rescue was seen upon injection of other cosmids from the region, B0504 (7 lines tested, <1% rescue) and C05H10 (10 lines tested, <1% rescue). *mgDf90* is a deletion that removes all of *daf-3*.

**Fig. 5B** shows the structure of the *C. elegans daf-3* coding region. The top is the exon/intron structure of *daf-3*; coding exons are filled boxes, non-coding regions are open boxes, and lines are introns. *daf-3* cDNAs were isolated according to standard methods. Four cDNAs were sequenced completely; their N-termini are indicated by vertical lines. These three cDNAs contain ~400 bp of 3' UTR, but no poly-A tail; a *C. elegans* consensus poly-adenylation sequence is found 12 bp from the 3' end of the cDNAs. The longest of this cDNA appears full-length, as it contains a methionine codon and the genomic sequence contains no other methionine codon and no putative splice sites upstream before in-frame stop codons. To further characterize the 5' end of *daf-3*, PCR products from libraries or individual *daf-3* cDNAs were sequenced. From DNA isolated from a cDNA library, we amplified a product with a primer to SL1 and to a region in conserved domain I (shown as primer 1). For the individual cDNAs, we amplified with a primer to the cDNA vector and primer 1. These PCR products were sequenced from primer 2 to the 5' end, and we found that there is alternative splicing at the 5' end of *daf-3*, upstream of the conserved domains. The two alternate splice forms are indicated, and the ends of individual cDNAs are indicated by vertical lines. Note that the second has the trans-spliced leader SL1 that is found at the 5' end of many *C. elegans* cDNAs; thus, this cDNA shows a *bona fide* 5' end of *daf-3*.

**Fig. 5C** shows the protein sequence alignment of *C. elegans daf-3* and the closest homolog found to date, human DPC4, in the Smad conserved domains I and II. Dots indicate gaps introduced to maximize alignment. DAF-3 is 55% identical to DPC4 in domain I and 30% identical in domain II. *daf-3(mg125)* and *daf-3(mg132)* mutations are indicated by boldface and underline. The Smad mutational hotspot is underlined. In addition to *mg125* and *mg132*, seven other *daf-3* alleles were sequenced in the hotspot; none of them contains a mutation. Alleles sequenced were *mg91*, *mg93*, *mg105*, *mg121*, *mg126*, *mg133* (isolated by A. Koweeck and G. Patterson, unpublished) and *sa205*.

**Figs. 6A-6G** is a panel of photographs showing *C. elegans* DAF-3 and DAF-4 expression. These photographs show GFP fluorescence, paired with DAPI fluorescence or Nomarski optics photographs, as marked. All DAF-3 photographs show animals with the second plasmid from **Fig. 6A** illustrates DAF-3/GFP head expression in an L1 animal. **Fig. 6B** illustrates DAF-3/GFP expression in the ventral nerve cord of an adult animal. L1 animals demonstrated similar expression patterns. **Fig. 6C** illustrates DAF-3/GFP expression in the intestine of an L1 animal. **Fig. 6D** illustrates DAF-3/GFP expression in the distal tip cell of an L4 animal. **Fig. 6E** illustrates DAF-3/GFP expression in an embryo with approximately 200 nuclei. **Fig. 6F** illustrates DAF-4/GFP expression in the head of an L1 animal. **Fig. 6G** illustrates DAF-4/GFP expression in the dorsal nerve cord and ventral nerve cord of an L4 animal.

**Fig. 7** is a table that shows the rescuing ability and suppression of *C. elegans daf-7* by *daf-3* plasmids. The solid boxes represent the Smad conserved domains I and II of *daf-3*; the stippled boxes represent green fluorescent protein (GFP). For all experiments shown, *daf-3* plasmids were injected at a concentration

of 10 ng/ml, and the pRF4 injection marker was injected at a concentration of 90 ng/ml. To score dauer formation, transgenic adult animals were allowed to lay eggs on plates for several hours at room temperature and were then removed. The plates were scored after two days at 25°C. The rescue experiment shows the rescue of *daf-7(m62); daf-3(e1376)* by each of the fusion proteins. Failure to rescue results in rolling nondauers, while rescue of *daf-3* results in rolling dauers (the *daf-7* phenotype). The control is an array with the pRF4 transformation marker and a non-rescuing cosmid. For each construct, four or more lines were measured in two separate experiments. To measure suppression of *daf-7*, transgenic arrays were crossed into *daf-7* (for plasmids 1 and 3), or produced by injecting directly into *daf-7* (for plasmid 2). Transgenic (rolling) animals were scored for suppression of *daf-7* (= nondauers) or failure to suppress *daf-7* (= dauers). The controls are two array strains with the pRF4 marker and an unrelated GFP expressing transgene.

**Fig. 8A** is a photographs showing that DAF-3/GFP is associated with metaphase chromosomes. Fixed L1 animals were immunostained with anti-GFP antibody and anti- $\alpha$ -tubulin antibody. DNA was visualized using DAPI staining.

**Fig. 8B** is a photograph showing that a truncated *C. elegans daf-3*/GFP protein is predominantly nuclear. Wild-type animals were injected with the truncated construct shown in **Fig. 7** at a concentration of 10 ng/ml. The pRF4 transformation marker was injected at 100 ng/ml. The photograph shows a late L1 or early L2 animal, and *daf-3* is predominantly nuclear. The clear spot in the center of some of the nuclei is the nucleolus, which has no *daf-3*/GFP. All cells in these animals have predominantly nuclear *daf-3*/GFP, including the ventral cord neurons, intestinal cells, and distal tip cell (all shown), as well as head and tail

neurons and hypodermal cells.

**Figs. 9A and 9B** show models for the role of the *C. elegans* *daf-3*/DAF-8/DAF-14 Smad proteins in dauer formation. **Fig. 9A** shows dauer reproductive growth induction. **Fig. 9B** shows reproductive dauer growth induction.

**Fig. 10** is a schematic illustration showing the genetic pathway that regulates *C. elegans* dauer formation.

**Figs. 11A-11C** show the cDNA sequences of the differentially spliced *C. elegans* *daf-3* transcripts (SEQ ID NOS: 39, 52, and 53).

**Figs. 12A-12C** show the amino acid sequences of the *C. elegans* DAF-3 polypeptide isoforms (SEQ ID NOS: 40-42).

**Figs. 13A and 13B** show the cDNA sequence of the differentially spliced *C. elegans* *daf-16* transcripts (SEQ ID NOS: 43 and 44).

**Figs. 14A and 14B** show the amino acid sequences of the *C. elegans* DAF-16 polypeptide isoforms (SEQ ID NOS: 45 and 46).

**Fig. 15** shows the cDNA sequence of the *C. elegans* *age-1* gene (SEQ ID NO: 47).

**Fig. 16** shows the amino acid sequence of the *C. elegans* AGE-1 polypeptide (SEQ ID NO: 48).

**Fig. 17** is a schematic diagram illustrating that convergent TGF- $\beta$  and insulin signaling activates glucose-based metabolic genes.

**Fig. 18** is a schematic diagram illustrating a switch to fat-based metabolism in the absence of DAF-7 and DAF-2 signals (in pheromone).

**Fig. 19** is a schematic diagram illustrating inhibition of the DAF-16 pathway by drugs to ameliorate lack of insulin signaling.

**Fig. 20** is a schematic diagram illustrating inhibition of DAF-3 by drugs to ameliorate a lack of DAF-7 signaling (for example in obesity-induced diabetes).

**Fig. 21A** (SEQ ID NOS: 211-215) is an illustration showing that human FKHR, FKHRL1, and AFX are the closest relatives to DAF-16. Note that the  
5 differentially spliced DAF-16 forkhead domain is less homologous.

**Fig. 21B** is an illustration showing a forkhead family tree, illustrating that DAF-16 is much more closely related to FKHR, FKHRL1, and AFX than any other forkhead protein.

**Fig. 22** is a photograph showing that *daf-16* is expressed in target  
10 tissues, like *daf-3*. This supports the model that DAF-3 and DAF-16 are capable of interacting.

**Fig. 23** is an illustration showing a model for treatment of obesity-induced diabetes with DAF-7 protein.

**Fig. 24** is an illustration showing the genetic mapping of *sup(mg144)* to  
15 the AKT genetic region.

**Fig. 25** is an illustration showing the comparison of *C. elegans* AKT with mammalian AKT.

**Fig. 26A** is a photograph showing the expression of AKT:GFP in *daf-2* dauers.

**Fig. 26B** is a photograph showing the expression of AKT:GFP in an N2 adult worm.

**Fig. 27** is a schematic illustration showing the molecular map of *daf-16*.

**Fig. 28** is a graph illustrating the homology of *C. elegans* insulin-like molecules (SEQ ID NOS: 117-124) with human insulin (SEQ ID NO: 125) and a  
25 consensus motif.

**Fig. 29** is a graph illustrating a PRETTYBOX analysis of insulin superfamily members (SEQ ID NOS: 126-153).

**Fig. 30** is a graph illustrating a PILEUP analysis of insulin superfamily members.

5        **Fig. 31** is a diagram illustrating the *akt-1* region. On the top is shown the genetic and physical map of *akt-1*. *akt-1* is contained on cosmid C12D8. Shown on the bottom is the exon/intron structure of *akt-1*. Coding regions are filled boxes, non-coding regions are open boxes, and introns are lines. The pleckstrin homology domain is indicated by hatched boxes (Musacchio et al.,  
10 Trends Biochem. Sci. 18:343-348, 1993). The kinase domain is indicated in gray (Hanks and Hunter, in The Protein Kinase Facts Book Protein-Serine Kinases, eds. Hardie, G. & Hanks, S., Academic Press, Inc., San Diego, CA, pp. 7-47, 1995). *akt-1a* gene structure was confirmed by sequencing of cDNAs. *akt-1b* gene structure was deduced based on partial cDNA sequence that confirmed the exon 5  
15 to exon 7 splice and 3'UTR only.

**Fig. 32** is a diagram illustrating the *akt-2* region. On the top is shown the genetic and physical maps of the *akt-2* region. *akt-2* is contained on cosmid R03E1. On the bottom is shown the exon/intron structure of *akt-2*. All symbols are as in **Fig. 31**. Gene structure was deduced by sequencing of a cDNA which  
20 confirmed exons 2-8 and the 3'UTR; Genefinder (Univ. of WA) predicts exon 1.

**Fig. 33** is a graph illustrating a dendrogram of Akt/PKB and PKC protein kinase families. Pileup (GCG) was used to align the entire coding sequences of the indicated proteins. *C. elegans* proteins are indicated by "Ce," rat by "r," human by "h," mouse by "m," bovine by "b," and *D. melanogaster* by "D." The  
25 accession numbers for the proteins used in the Pileup are contained in parentheses:

CePKC2a(U82935), rPKC $\beta$ 1(M19007), hAkt/PKB $\alpha$ (M63167),  
 mAkt/PKB(M94335), bAkt/PKB(X61036), hAkt/PKB $\beta$ 2(M95936),  
 rAkt/PKB $\gamma$ (D49836), Dakt1(Z26242). To anchor the tree, rPKC $\beta$ 1 (the closest  
 non-Akt/PKB homolog to both *akt-1a* and hAkt/PKB $\alpha$ ), and CePKC2a (the closest  
 5 *C. elegans* homolog to rPKC $\beta$ 1) were included in the Pileup. The Akt/PKB  
 homologs described in this report are indicated by the gray box.

**Fig. 34** is a graph illustrating a PILEUP (GCG) analysis of AKT-1a  
 (SEQ ID NO: 154), AKT-1b (SEQ ID NO: 155), AKT-2 (SEQ ID NO: 156), and  
 human Akt/PKB $\alpha$  (M63167) (SEQ ID NO: 157). Identical residues are indicated  
 10 by dots, gaps introduced in order to align the sequence are indicated by dashes.  
 The pleckstrin homology domain (Musacchio et al., Trends Biochem. Sci. 18:343-  
 348, 1993) is indicated by the N-terminal gray shaded areas, the kinase domain  
 (Hanks and Hunter, in The Protein Kinase Facts Book Protein-Serine Kinases, eds.  
 Hardie, G. & Hanks, S., Academic Press, Inc., San Diego, CA, pp. 7-47, 1995) is  
 15 indicated by the C-terminal gray shaded areas. The mg144 Ala183Thr substitution  
 is indicated as a T above the AKT-1a sequence. The Akt-1 and AKT-2  
 phosphorylation sites that correspond to the hAkt/PKB $\alpha$  Thr308 and Ser473  
 phosphorylation sites (Alessi et al., EMBO J. 15:6541-6551, 1996) are indicated as  
 dots above the amino acid residue that is phosphorylated.

20 **Figs. 35A and 35B** show the genomic sequence of *pdk-1* (SEQ ID NO:  
 158).

**Fig. 36** shows the amino acid sequence of *pdk-1a* (SEQ ID NO: 159).

**Fig. 37** shows the amino acid sequence of *pdk-1b* (SEQ ID NO: 160).

**Figs. 38A-38F** show metabolic control by *age-1* and *daf-18*. Fat  
 25 accumulation was assayed by Sudan Black staining in hermaphrodites grown at

20°C. The animal in Fig. 38E is a dauer larva, whereas Figs. 38A-D and F are comparable reproductive larval stage 4 animals. Fig. 38A shows a wild type (Bristol N2) animal. Fig. 38B shows a *daf-18(e1375)* animal. Fig. 38C shows an *age-1(mg44)/mnC1* animal. This L4 stage larva has both maternal and zygotic contributions of *age-1*. Fig. 38D shows an *age-1(mg44)* animal. This L4 stage larva is a homozygote from an *age-1(mg44)/mnC1* parent and has a maternal, but not zygotic, contribution of *age-1*. This maternal contribution of *age-1* is sufficient to allow reproductive development, but the animal accumulates larger amounts of fat than the wild type or the zygotically rescued *age-1* mutant. Fig. 38E shows an *age-1(mg44)* animal. This dauer larva is a progeny of a maternally rescued *age-1(mg44)* animal. The lack of maternal and zygotic contribution of *age-1* causes this animal to develop as a dauer and accumulate fat. Fig. 38F shows an *age-1(mg44); daf-18(e1375)* animal. This L4 stage larva lacks both a maternal and zygotic contribution of *age-1*, but does not develop into a dauer due to the suppression by the *daf-18* mutation. The *daf-18* mutation also suppresses the accumulation of fat phenotype of the *age-1* null mutant.

**Figs. 39A and 39B** illustrate that *daf-18* encodes a homologue of PTEN (MMAC/TEP1). Fig. 39A shows the exon/intron structure of DAF-18 (SEQ ID NO: 365-368). The phosphatase domain is indicated in gray. The bottom of this figure indicates that *daf-18(e1375)* has a 30 base pair insertion in the fourth exon. 13 base pairs (shaded) are duplicated along with two smaller segments of the repeat (thick bars). This mutation introduces a premature stop codon (\*). Fig. 39B shows an alignment of the phosphatase domains of DAF-18 and PTEN (GeneBank accession U93051) (SEQ ID NO: 369-378). Pileup (GCG) was used to align the entire coding sequence. The phosphatase domain is shown with identical amino

acids shaded. The probable active site Cys-(X)<sub>5</sub>-Arg sequence is indicated with a bar.

**Figs. 40A and 40B** show the amino acid and nucleic acid sequences of the *C. elegans daf-18* gene (SEQ ID NO: 379-380).

5           **Fig. 41** illustrates a model for the regulation of metabolism and dauer arrest by insulin receptor-like signaling. DAF-2 insulin receptor-like activates AGE-1 PI3K to generate PIP<sub>3</sub> and PI(3,4)P<sub>2</sub>. PIP<sub>3</sub> and PI(3,4)P<sub>2</sub> may activate AKT-1 and AKT-2 directly by binding to the PH domain and indirectly by regulating PDK1-mediated phosphorylation of the threonine 308 equivalent site.

10 In addition, AKT-1 may be regulated by phosphorylation at the serine 473 equivalent (AKT-2 lacks this site). DAF-18 PTEN limits AGE-1 PI3K signals by dephosphorylating PIP<sub>3</sub> and/or PI(3,4)P<sub>2</sub>. In the absence of AGE-1 signals, loss of DAF-18 allows an alternative source of PI(3,4)P<sub>2</sub> and PIP<sub>3</sub> to accumulate and activate AKT-1 and AKT-2. AKT-1/AKT-2 signals converge with an additional

15 signaling pathway from the DAF-2 receptor to regulate the DAF-16 Fork head transcription factor. DAF-16 responsive genes control metabolism, reproductive growth, and lifespan.

**Fig. 42** shows the *C. elegans cod-5* nucleic acid and amino acid sequences (SEQ ID NO: 381-382).

20           **Figs. 43** shows the *C. elegans cod-5* knockout cDNA and amino acid sequences (SEQ ID NO: 383-384).

**Figs. 44A, 44B, and 44C** show the effect of muscarinic agonists and an antagonist on dauer recovery in *C. elegans* and *A. caninum*. In Figure 44A, oxotremorine, a synthetic muscarinic agonist, promotes dauer recovery in both *C.*

25 *elegans* and *A. caninum*. Note that *daf-2(e1370)* fails to recover at all

concentrations. In Figure 44B, arecoline, a natural muscarinic agonist, promotes dauer recovery in both *C. elegans* and *A. caninum*. Note that *daf-2(e1370)* fails to recover at all concentrations. Figure 44C shows that atropine can specifically inhibit the muscarinic agonist-induced response. In *C. elegans*, at 1 mM oxotremorine, as the concentration of atropine, a muscarinic antagonist, is increased, dauer recovery is completely inhibited. Similarly, in *A. caninum* larvae, arecoline and increasing amounts of atropine cause dauer recovery to be completely inhibited.

**Figs. 45A and 45B** show that atropine specifically inhibits dauer recovery in *C. elegans* and *A. caninum*. In Figure 45A, wild-type N2 dauers were placed on plates containing either bacterial food; no bacteria and no pheromone; bacteria and 1 mM atropine; or pheromone at 25 degrees. 42 hours later, the plates were scored for the presence of dauers and reproductive L4/adults. With no food and no pheromone, 100% of the animals remained arrested at the dauer stage (n>280). Addition of food caused efficient dauer recovery at 25 degrees. Dauers placed on plates with food recovered efficiently, with less than 1% remaining arrested at the dauer stage (n>1000). Addition of 1 mM atropine in the presence of food inhibited dauer recovery: 82% remained arrested at the dauer stage (n=1432). 80% of the animals maintained on plates with pheromone but no food (n=505) remained arrested at the dauer stage. The pheromone preparation contained bacterial contaminants that may have been used as a food source. In *A. caninum* incubated with 10% serum and 25 mM GSM, 9% of the infective larvae remained as dauers and did not resume feeding. Addition of atropine (0.5 mM) to the serum and GSM completely inhibited recovery of *A. caninum* L3 and no worms resumed feeding. In Figure 45B, *daf-2(e1370)* and *daf-7(e1372)* dauers were placed onto

plates at 15 degrees. Animals were scored for the presence of dauers and reproductive adults two days after food was added to the plate. Bacterial food was added after temperature downshift failed to induce dauer recovery in *daf-2(e1370)* (n=140) and *daf-7(e1372)* (n=36). Only 21% of the *daf-2(e1370)* (n=509) and 21% of the *daf-7(e1372)* (n=112) dauer larvae on plates at the lower temperature with food remained as dauers after two days. Atropine at 1 mM completely inhibited dauer recovery on *daf-2(e1370)* (n=205) and *daf-7(e1372)* (n=166) dauers on plates at 15 degrees in the presence of food.

**Fig. 46** shows a model for cholinergic input induction of dauer recovery. In dauer pheromone or in a *daf-7* mutant, the DAF-7 TGF- $\beta$  ligand is not produced by the ASI sensory/secretory neuron. Therefore, there is no activation of the DAF-1 and DAF-4 TGF- $\beta$  receptors or downstream DAF-8 and DAF-14Smad proteins, and this results in high DAF-3 Smad activity in target tissues. In pheromone without muscarinic agonists, no insulin like signal is released, causing a lack of DAF-16 regulation, which in combination with unregulated DAF-3 induces dauer arrest. Under these conditions, muscarinic stimulation causes release of an insulin-like DAF-2 ligand which stimulates the DAF-2/AGE-1 signaling pathway to DAF-16 activation in target tissues. Since *daf-7* mutants can recover in muscarinic agonists, the TGF- $\beta$  signaling pathway is not required for dauer recovery.

Under normal conditions of dauer recovery upon release from pheromone and addition of food and low temperature, these conditions may cause release of acetylcholine, either through the temperature or food pathways, which binds to the muscarinic receptor on the insulin-like signaling cell. Binding of acetylcholine to the receptor causes an increase in insulin release. Temperature may be coupled via

the interneurons AIY and AIZ to the DAF-2 insulin-like signaling pathway, rather than the TGF- $\beta$  signaling pathway, because mutations in the thermoregulatory gene *ttx-3* can suppress mutations in *daf-7* and not mutations in *daf-2*.

**Figs. 47A and 47B** show the nucleic acid and amino acid sequences of a  
5 human DAF-7 homologue (SEQ ID NO: 385-386).

**The DAF-2 Insulin Receptor Family Member Regulates Longevity and  
Diapause in *C. elegans***

Arrest at the *C. elegans* dauer stage is normally triggered by a  
dauer-inducing pheromone detected by sensory neurons which signal via a  
10 complex pathway to target tissues that are remodeled and metabolically shifted  
such as the germ line, intestine, and ectoderm (Riddle, In: *Caenorhabditis elegans*  
II, D. Riddle, T. Blumenthal, B. Meyer, J. Priess, eds., Cold Spring Harbor  
Laboratory, Cold Spring Harbor, NY, 1997, pp. 739-768. Kenyon, op cit., pp.  
791-813.). Genetic epistasis analysis of *daf* mutants that arrest at the dauer stage  
15 or enter the reproductive life cycle independent of pheromone regulation has  
revealed parallel genetic pathways that regulate distinct aspects of the dauer  
metamorphosis (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and  
Ruvkun, *Genetics* 137: 107-120, 1994). The pathway that includes *daf-2* is  
unique in that it controls both reproductive development and normal senescence:  
20 *daf-2* mutant animals arrest development at the dauer larval stage and have  
dramatically increased longevity (Table I) (Riddle, In: *Caenorhabditis elegans* II,  
D. Riddle, T. Blumenthal, B. Meyer, J. Priess, eds., Cold Spring Harbor  
Laboratory, Cold Spring Harbor, NY, 1997, pp. 739-768; Kenyon, op cit. pp  
791-813; Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and

Ruvkun, *Genetics* 137: 107-120, 1994; Larsen et al., *Genetics* 139: 1567-1583, 1995; Kenyon et al., *Nature* 366: 461-464, 1993; Dorman et al., *Genetics* 141: 1399-1406, 1995).

Table I shows the percentage of dauer formation of *daf-2* alleles and the associated mutations. Eggs from animals grown at 15°C (day 0) were incubated at 15, 20, or 25°C. Numbers in parenthesis are animals counted. Numbers of wild-type animals and dauers were counted on day 3 (20°C and 25°C) or day 5 (15°C). Most of the dauers marked with stars recovered by day 4 (*sa229* at 25°C) or by day 8 (*sa229*) and *sa219* at 15°C, *el368* and *sg219* at 20°C, and *el365* and *el368* at 25°C). *mg43* was studied as follows: *dpy-1(el)daf-2(mg43); SDP3* animals were grown at 20°C until the young adult stage. Eggs from five adults were laid at 15°C or 20°C and grown at the same temperatures. Numbers of Dpy-Daf animal and Dpy-non-Daf animals were counted on day 3 (20°C) or day 5 (15°C). *Sg187* and *sg229* were also studied by Malone and Thomas (*Genetics* 136:879-886, 1994).

Table I. Percentage of dauer formation of *daf-2* alleles

Region	Allele	mutation	% dauer formation		
			15°C	20°C	25°C
cys-rich	mg43	C279Y&P348L	100.0 (215)	100.0 (245)	n.d.
	sa187	C347S	0.4 (461)	98.7 (224)	100 (910)
ligand-binding	e1368	S451L	0.0 (328)	4.5* (418)	99.7* (698)
	e1365	A458T	0.0 (450)	0.0 (461)	99.4* (814)
	sa229	D526N	3.4* (234)	n.d.	22.1* (420)
	sa219	D1252N	10.0* (460)	99.7* (396)	100 (514)
kinase					

		% dauer formation		
e1391	P1312L	3.3 (332)	100 (323)	100 (322)
e1370	P1343S	0.0 (520)	0.0 (188)	100 (635)

Genetic mapping using both visible genetic markers and restriction fragment length polymorphism (RFLP) markers places *daf-2* between *mgP34* and *mgP44* (Fig. 1). While cosmid coverage of this physical genetic region is not complete, YAC Y53G8 carries the genomic region that includes *mgP34* and *mgP44*, which flank *daf-2* (Fig. 1). As a step in the *C. elegans* genome sequencing effort, random M13 subclones derived from Y53G8 were sequenced by the Genome Sequencing Center.

**Sequence Identities Show that DAF-2 is Likely to Bind to an Insulin-like Ligand and to Phosphorylate Tyrosine Residues**

The amino acid sequences and nucleotide sequences encoding DAF-2 are shown in Figs. 2A and 2B, respectively. Using BLASTX to compare 570 translated Y53G8 M13 subclone sequences against the Genbank protein database, we found that four sequences are homologous to the mammalian insulin receptor family. An insulin receptor was a good *daf-2* candidate gene because insulin regulates vertebrate growth and metabolism (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994), and because a phosphatidylinositol (PI) 3-kinase has been shown to act in both the insulin receptor and *daf-2* pathways (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994; Morris et al., *Nature* 382: 536-539, 1996). The detection of multiple *daf-2* mutations in the gene (see below), and the coincidence of the

genetic location of this insulin receptor homolog with *daf-2* (Fig. 2C) establish that this insulin receptor homolog corresponds to *daf-2*.

The *daf-2* transcription unit and gene structure were determined using PCR primers derived from *daf-2* genomic subclone sequences to amplify *daf-2* genomic and cDNA regions. A probable full length *daf-2* cDNA bears a 5172  
5 base open reading frame, a 485 base 5' UTR and a 159 base 3' UTR (Figs. 1, 2A). The predicted DAF-2 protein shows long regions of sequence identity to the insulin receptor family. Over the entire protein, DAF-2 is 35% identical to the human insulin receptor (Ebina et al., *Cell* 40: 747-58, 1985; Ullrich, et al., *Nature*  
10 313: 756-61, 1985), 34% identical to the human IGF-I receptor (Ullrich, et al., *EMBO J.*: 5, 2503-12, 1986), and 33% identical to the human insulin receptor-related receptor (Shier and Watt, *J. Biol. Chem.* 264: 14605-8, 1989). DAF-2 is the only member of the insulin receptor family in the 90 Mb *C. elegans* genome sequence (about 90% complete) or in the 10 Mb *C. elegans* EST sequence  
15 database. Because it is equally distant from insulin, IGF-I, and insulin receptor-related receptors, DAF-2 is probably the homolog of the ancestor of these duplicated and diverged receptors, and thus may subserve any or all of the functions of these mammalian receptors (see below). Like these receptors, DAF-2 has a putative signal peptide, a cysteine-rich region in the putative ligand binding  
20 domain, a putative proteolysis site, a transmembrane domain, and a tyrosine kinase domain. In addition, DAF-2 has a C-terminal region that may serve a function similar to the mammalian insulin receptor substrate-1 (IRS-1) (Figure 2; White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994).

In the approximately 500 amino acid ligand-binding domain of the  
25 insulin receptor, DAF-2 is 36% identical to insulin receptor and 35% identical to

the IGF-I receptor. Twenty-one of twenty-three phylogenetically conserved cysteine residues in this domain are conserved in DAF-2 (Fig. 2C). The DAF-2 cys-rich region is 34% identical to human insulin receptor and 28% identical to the IGF-I receptor. Six *daf-2* mutations map in this domain (Fig. 2C, Table I). The *mg43* and *sal87* mutations substitute conserved residues in the cys-rich region (Fig. 2C). *daf-2(mg43)* carries two mutations which substitute conserved residues, which may explain the strength of this allele (non-conditional, Table I). Other substitutions at non-conserved residues cause less severe phenotypes (Table I). Insulin resistant and diabetic patients with mutations in the ligand binding domain of the human insulin receptor gene have been identified (Taylor, *Diabetes* 41: 1473-1490, 1992) (see below). These mutations impair receptor transport to cell surface, or insulin binding affinity, or both. The DAF-2 mutations in this domain might similarly decrease receptor signaling to cause dauer arrest.

Insulin receptors are  $\alpha 2, \beta 2$  tetramers proteolytically processed from a single precursor protein (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). DAF-2 bears a probable protease recognition site at a position analogous to the insulin receptor processing site (RVRR 806-809) (Yoshimasa et al., *J. Biol. Chem.* 265: 17230-17237, 1990).

The 275 amino acid DAF-2 tyrosine kinase domain is 70% similar and 50% identical to the human insulin receptor kinase domain. Upon insulin binding, the intracellular tyrosine kinase domain of the insulin receptor phosphorylates particular tyrosine residues flanked by signature amino acid residues (upstream acidic and downstream hydrophobic amino acids (Songyang and Cantley, *Trends Biochem. Sci.* 20: 470-475, 1995)) in the intracellular domain as well as on IRS-1 (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). Multiple DAF-2 tyrosine

residues in these sequence contexts are likely autophosphorylation targets, including three tyrosines in a region similar to the insulin receptor activation loop and one in the juxtamembrane region as described above (Fig. 2C). Based on the crystal structure of the insulin receptor kinase domain bound to its activation loop, eight kinase domain residues mediate target site specificity (Hubbard et al., *Nature* 372: 746-754, 1994). In DAF-2 (but not in more distantly related receptor kinases), these residues are invariant (5/8) or replaced with similar amino acids (3/8: K to R, E to D) (Fig. 2C), suggesting that DAF-2 phosphorylates the same target tyrosine motifs as the insulin receptor kinase.

Three *daf-2* missense mutations substitute conserved amino acid residues in the kinase domain (Fig. 2C, Table I). All three mutations cause moderate to strong dauer constitutive phenotype, but none are as strong as the non-conditional alleles, for example, *mg43* (Table I). Human insulin receptor mutations in the kinase domain exhibit decreased kinase activity and cause severe insulin resistance and associated defects (Fig. 2C; Taylor, *Diabetes* 41: 1473-1490, 1992). Remarkably, a human diabetic insulin resistant patient bears the same amino acid substitution (P1178L) as *daf-2(e1391)* (Kim et al., *Diabetologia* 35: 261-266, 1992). This patient was reported to be heterozygous for this substitution. *daf-2(e1391)* is not dominant whereas it is a highly penetrance recessive mutation (Table I).

To test for dominance of *daf-2(e1391)*, using a genetically marked balancer chromosome, 105 dauers segregated from 485 *daf-2/+* parents as expected for a recessive mutations. The genotype of 76/77 of these animals was homozygous *daf-2(e1391)* whereas 1/77 of the dauers was *daf-2(e1391)/+*, indicating a less than 1% dominance. It is possible that in contrast to *C. elegans*,

the P1178L mutation in humans is dominant, or that the patient carries a second insulin receptor mutation in *trans*, or carries mutations in other genes (for example, other complex type II diabetes loci) that enhance the dominance of P1178L (Bruning et al., *Cell* 88: 561-572, 1997).

## 5 *AGE-1 PI 3-kinase is a Major DAF-2 Signaling Output*

Like the *Drosophila* insulin receptor homolog, DAF-2 has a long C-terminal extension that may function analogously to mammalian IRS-1 (Fernandez et al., *EMBO J.* 14: 3373-3384, 1995). In mammals, IRS-1 tyrosine residues are phosphorylated by the insulin receptor kinase, and these  
 10 phosphotyrosines mediate binding to a variety of signaling proteins bearing SH2 domains (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994; Songyang et al., *Cell* 72: 767-778, 1993.). Many, but not all, of the DAF-2 C-terminal extension tyrosines bear flanking sequence motifs suggestive that they are autophosphorylated (Fig. 2A; Songyang and Cantley, *Trends Biochem. Sci.* 20: 470-475, 1995). Based on  
 15 precedents from IRS-1 interactions with mammalian PI 3-kinases (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994), a YXXM motif at DAF-2 Y1504 is likely to mediate interaction with the AGE-1 PI 3-kinase, which acts in the same genetic pathway as *daf-2* (Fig. 4) (Morris et al., *Nature* 382: 536-539, 1996).

Three DAF-2 tyrosine residues, Y1293, Y1296 and Y1297, in the region  
 20 corresponding to the insulin receptor kinase Y1158 to Y1163 activation loop are likely to be autophosphorylated, based on the predicted similarity between the DAF-2 and insulin receptor phosphorylation targets (Fig. 2C). Another likely target for DAF-2 autophosphorylation is the Y1106 NPEY motif located in the region corresponding to the insulin receptor juxtamembrane region NPEY motif

(at Y972), that has been shown to mediate IRS-1 binding via its PTB domain to the insulin receptor (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). While DAF-2 bears one YXXM motif implicated in coupling to PI 3-kinase, mammalian IRS-1 and *Drosophila* insulin receptor (Fernandez et al., *EMBO J.* 14: 3373-3384, 1995) bear multiple YXXM motifs. Although no p85-like adaptor subunit has yet been detected in the *C. elegans* database, the AGE-1 homology to mammalian p110 suggests the existence of a homologous or analogous adaptor (Morris et al., *Nature* 382: 536-539, 1996). In the DAF-2 C-terminal domain, two other tyrosine residues may be autophosphorylated and bound to particular SH2-containing proteins: Y1678 binding to a PLC- $\gamma$  or SHP-2 homolog, and Y1686, perhaps binding to SEM-5 (Fig. 2A) (Songyang et al., *Cell* 72: 767-778, 1993). While mutations in, for example, ras and MAP kinase have not been identified in screens for dauer constitutive or dauer defective mutations, these general signaling pathway proteins may couple to DAF-2 as they couple to insulin signaling in vertebrates (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994).

The insulin receptor also couples to other signaling pathways (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994); analogous DAF-2 phosphotyrosine residues may mediate these interactions (as described above). Thus, we suggest that tyrosines in the DAF-2 cytoplasmic domain are autophosphorylated upon ligand binding, and recruit the AGE-1 PI-3 kinase homolog (as well as other molecules) to signal reproductive development and normal senescence.

### **Metabolic Control by daf-2 in Control of Diapause and Aging**

Insulin and its receptor families play key roles in vertebrate (and by our evidence in invertebrates) metabolic and growth control (Kahn and Weir, eds.,

Joslin's Diabetes Mellitus, Lea & Febiger, 1994). Upon insulin release--by increasing blood glucose and autonomic inputs--insulin receptor engagement directs a shift in the activities of key metabolic enzymes, as well as changes in the transcription and translation of metabolic regulators in fat, liver, and muscle cells, all of which lead to assimilation of glucose into glycogen and fat (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). IGF-I is released from the liver in response to pituitary growth hormone, and mediates many of the growth and development responses to that endocrine signal (Mathews et al., *Proc Natl Acad Sci. U.S.A.* 83: 9343-7, 1986). Interestingly, lifespan is dramatically increased in dwarf mice with defects in growth hormone signaling, and presumably decreased IGF-I signaling as well (Brown-Borg et al., *Nature* 384: 33, 1996). No function for the insulin receptor-related receptor has yet been established, though it is expressed in conjunction with NGF receptor (Reinhardt et al., *J. Neurosci.* 14: 4674-4683, 1994).

Diapause arrest in general and dauer arrest in particular are associated with major metabolic changes (Tauber et al., *Seasonal Adaptation of Insects*, Oxford University Press, New York, N. Y., 1986), consistent with a model that *daf-2* acts in a metabolic regulatory pathway related to insulin signaling. In wild-type animals, DAF-2 signaling allows non-dauer reproductive growth, which is associated with utilization of food for growth in cell number and size, and small stores of fat (Fig. 3). In *daf-2* mutant animals, metabolism is shifted to the production of fat (Fig. 3) and glycogen (data not shown) in intestinal and hypodermal cells. Even when a temperature-sensitive *daf-2* mutant allele is shifted to the non-permissive temperature at the L4 or adult stage (after the critical period for *daf-2* control of dauer formation), metabolism is shifted towards storage of fat

(Fig. 3). Thus *daf-2* also regulates metabolism during reproductive development. Similar metabolic shifts are seen in wild-type pheromone-induced dauers (data not shown), *age-1* mutants (data not shown), and *daf-7* mutants (Fig. 3). In support of this metabolic shift, in dauer larvae, enzymes that regulate glycolysis are

5 down-regulated while those that regulate glycogen and fat synthesis are up-regulated, and there is ultrastructural evidence for increased lipid and glycogen (O'Riordan and Burnell, *Comp. Biochem. & Physiol.* 92B: 233-238, 1989; O'Riordan and Burnell, *Comp. Biochem. & Physiol.* 95B: 125-130, 1990; Popham and Webster, *Can. J. Zool.* 57: 794-800, 1978; Wadsworth and Riddle, *Develop.*

10 *Biol.* 132: 167-173, 1989). The dauer metabolic shift is associated with arrest of germ line proliferation, and arrest of somatic cell division and enlargement (Riddle, In: *Caenorhabditis elegans* II, D. Riddle, T. Blumenthal, B. Meyer, J. Priess, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1997, pp. 739-768; Kenyon, op cit., pp. 791-813).

15           There is precedent for insulin-like signaling in invertebrate metabolic and growth control: insulin-like growth factors have been detected in metabolism-regulating ganglia in molluscs (Roovers et al., *Gene* 162: 181-188, 1995) and regulate molting in locust (Hetru et al., *Eur. J. Biochem* 201: 495-499, 1991) and silkworm (Kawakami et al., *Science* 247: 1333-1335, 1990).

20 Consistent with the *daf-2* regulation of diapause, injection of insulin into diapausing *Pieris brassicae* (an insect) pupae induces recovery (Arpagaus, *Roux's Arch. Dev. Biol.* 196: 527-530, 1987).

Without being bound to a particular theory, we hypothesize that an insulin-like signal is up-regulated during reproductive development and stimulates

25 DAF-2 receptor autophosphorylation and recruitment of the AGE-1 PI 3-kinase to

produce the second messenger PIP3. AGE-1 is likely to be a major signaling output of DAF-2 because of the similarity of the *age-1* and *daf-2* mutant phenotypes and because of their similar placement in the epistasis pathway (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994). Precedents from insulin receptor signaling suggest the following candidate targets for DAF-2/AGE-1/PIP3 regulation of metabolism: (1) membrane fusion of vesicles bearing glucose transporters (Kahn and Weir, eds., *Joslin's Diabetes Mellitus*, Lea & Febiger, 1994) (or more probably trehalose transporters (Tauber et al., *Seasonal Adaptation of Insects*, Oxford University Press, New York, N. Y., 1986)) to facilitate flux of this molecule for growth and reproductive metabolism; (2) PIP3 activates an AKT/GSK-3 kinase cascade (Hemmings, *Science* 275: 628-630, 1997) which may regulate the activities of glycogen and fat synthetic and lytic enzymes; (3) transcription and translation of metabolic genes such as PEPCK, GDH, fat synthetases, and lipases (White and Kahn, *J. Biol. Chem.* 269:1-4, 1994). Genetic epistasis analysis suggests that DAF-2/AGE-1 signaling negatively regulates *daf-16* gene activity (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994). DAF-16 could act at any point downstream of AGE-1 in this signaling pathway. Evidence is presented herein that DAF-16 represents the major transcriptional output to DAF-2/AGE-1 PIP3 signaling.

In addition to these metabolic changes, the DAF-2 signaling cascade also controls the reproductive maturation of the germ line as well as morphogenetic aspects of the pharynx and hypodermis (Riddle, In: *Caenorhabditis elegans* II, D. Riddle, T. Blumenthal, B. Meyer, J. Priess, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1997, pp. 739-768; Kenyon, op cit., pp.

791-813). The DAF-2 receptor may act, for example, in the hypodermal and intestinal target tissues where we note a change in metabolism triggered by the dauer regulatory cascade (Fig. 3). It is also possible that DAF-2 regulates the metabolism and remodeling of tissues indirectly, for example, by controlling the  
5 production of other hormones (Nagasawa et al., *Science* 226: 1344-1345, 1984; Jonas, et al., *Nature* 385: 343-346, 1997). Expression and genetic mosaic analysis of *daf-2* is essential to distinguish these models.

Even though DAF-2 and the mammalian insulin receptor both regulate metabolism, the metabolic defects associated with mutations in these receptors  
10 appear to be different. Complete loss of mammalian insulin receptor activity causes growth arrest at birth (Leprechaunism in humans), and a metabolic shift to runaway lipolysis and ketoacidosis (Kahn and Weir, eds., *Joslin's Diabetes Mellitus*, Lea & Febiger, 1994), rather than the fat accumulation we observe in *daf-2* mutants (Fig. 3). This distinction between insulin receptor and *daf-2*  
15 mutants may reflect distinct metabolic responses to this signaling, or a difference between complete loss and declines in insulin signaling. In humans, ketoacidosis is only induced during severe starvation or pathological states when insulin levels are very low (Kahn and Weir, eds., *Joslin's Diabetes Mellitus*, Lea & Febiger, 1994). Since none of the *daf-2* mutations described herein are clear null  
20 mutations, it is possible that *daf-2* dauer-constitutive alleles are more analogous to non-null human insulin receptor mutations. Most *daf-2* alleles are temperature sensitive, including alleles isolated in genetic screens that would allow the recovery of non-temperature sensitive mutations (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994).  
25 Substitutions of DAF-2 amino acid residues conserved across phylogeny cause

more penetrant dauer arrest at all temperatures than substitutions of non-conserved residues. *daf-2* mutants that arrest development at the dauer stage independent of growth temperature are likely to have the least gene activity (for example *mg43*). Several *daf-2* alleles also cause 5% to 10% embryonic lethality (unpublished results), suggesting that *daf-2* functions during embryonic development. None of the *daf-2* mutations detected so far are nonsense, frameshift, or deletion alleles. It is possible that the *daf-2* null phenotype is stronger than non-conditional dauer arrest, for example embryonic lethality. However, dauer constitutive *daf-2* mutant alleles are isolated from EMS mutagenesis at a very high rate (about 1/300 chromosomes), suggesting that the existing alleles are not rare viable alleles. In fact, the 14 year old patient with the same insulin receptor mutation as *daf-2(e1391)* was morbidly obese (Kim et al., *Diabetologia* 35: 261-266, 1992), suggesting that metabolic effects of decreased insulin signaling may be similar to *daf-2* mutants.

It may be significant to human diabetes that animals carrying mutations in *daf-16* can grow reproductively even if they also carry *daf-2* and *age-1* mutations that disable insulin-like metabolic control signals (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994). These data suggest that it is unregulated *daf-16* gene activity that causes these metabolic shifts. The analogous metabolic defects associated with both type I and type II diabetes may be caused by similar unregulated activity of the human DAF-16 homolog. Below we disclose the molecular identity of *daf-16*. Inhibition of its activity is expected to ameliorate the metabolic dysregulation associated with insulin signaling defects.

### **DAF-16 Encodes a Forkhead Transcription Factor Homolog**

Using a combination of genetic mapping and detection of multiple *daf-16* mutations in a 5 kb region, we have determined the nucleic acid sequence of *daf-16*. *daf-16* was mapped 1 map unit to the left of *lin-11* and 3.3 map units right of *unc-75* on Chromosome I. This region of the genome contained a gap that was not covered by cosmids nor YACs. We used a fosmid library (Genome Sciences, Inc.) to walk into the gap. Sequence analysis of the ends of four fosmids (H27K20, H01H03, H12I08, and H35K06) revealed that the previously unmapped contig 133 lies in the *lin-11 unc-75* gap. Cosmids from the approximate *daf-16* genetic location were used to detect RFLPs between *C. elegans* strains Bristol N2 and Bergerac RC301: *mgP45* on cosmid C39H11, *mgP46* on cosmid F28D9, *mgP49* on cosmid C35E7, *mgP50* is on cosmid C43H8. Zero out of 30 *daf* non-Unc recombinants carry the RC301 alleles of *mgP45* and *mgP50*. Two out of 30 *Daf* non-Unc recombinants carry the RC301 allele of *mgP49*. 10 out of 30 *Daf* non-Unc recombinants carry the RC301 allele of *mgP46*. 1 out of 4 non-Lin *Daf* recombinants carry the N2 allele of *mgP45*. 4 out of 4 non-Lin *Daf* recombinants carry the N2 allele of *mgP49*. These data indicate that *daf-16* lies between cosmids C43H8 and C35E7. The *daf-16* gene was identified by identifying deletions (*mgDf50*) and point mutations (*mg53* and *mg54*) within the forkhead gene on the cosmid R13H8 (Fig. 27). There are two major *daf-16* transcripts whose sequences are shown in Fig. 13A and Fig. 13B (SEQ ID NOS: 43 and 44, respectively). The amino acid sequences coding for the DAF-16 isoforms are shown in Figs. 14A-14C (SEQ ID NOS: 44-46).

We have detected three *daf-16* mutations: (1) a large deletion of conserved regions in *daf-16* (*mg ΔF50*) that proves that the *daf-16* null phenotype

is a suppression of *daf-2* mutations; (2) a S to L substitution in exon 6 in *daf-16* (mg 53) that alters a conserved WKNSIRH motif; and (3) a nonsense mutation in exon 3 in *daf-16* (mg 54) that is predicted to truncate one of the *daf-16* differentially spliced isoforms. Interestingly, this spliced isoform has a distinct forkhead DNA binding domain and is therefore expected to bind to distinct promoters or combinatorial partners. This mutant is a weak suppressor of *daf-2*, suggesting that both DAF-16 isoforms are necessary for metabolic control.

Sequence analysis has revealed that DAF-16 is a member of the forkhead (FH) transcription factor family (Figs. 21A-21B). This strong amino acid homology indicates that DAF-16 is a transcription factor. Our genetic analysis indicates that DAF-16 activity is regulated by the DAF-2/AGE-1 insulin signaling pathway. Precedent from another receptor kinase signaling pathway endorses this model: the *C. elegans* LIN-31 forkhead protein has been shown to be regulated by a tyrosine kinase signaling cascade from the LET-23 EGF receptor homolog (Kim, *Genes Dev.* 7: 933-947, 1993). Consistent with a model that DAF-16 acts downstream of insulin signaling, forkhead transcription factors have also been implicated in metabolic regulation: another FH family member is mammalian HNF-3, an endoderm-specific transcription factor that acts at the same metabolic control protein promoters as HNF-1 and HNF-4, both of which are mutant in maturity onset diabetes of the young (MODY) (Yamagata et al., *Nature* 384: 455-458, 1996; Yamagata et al., *Nature* 384: 458-460, 1996).

The identification of DAF-16 as a forkhead transcription factor also explains much of the complex *daf* genetics of *C. elegans*. The convergence of DAF-7 TGF- $\beta$ -like signaling and DAF-2 insulin-like signaling is also explained by our discovery that DAF-16 is a FH protein and DAF-3 is a Smad protein:

Precedent for an interaction between Smad and forkhead proteins has been found in *Xenopus*. Response to the TGF- $\beta$  superfamily relative activin in early frog development is mediated by an interaction between the distant relative of DAF-16 called FAST-1, and the Smad protein, Smad2 (*Nature* 383: 600-608, 1996). These proteins bind to an enhancer element that is very similar to the myosin II promoter to which DAF-3 binds (see below). Thus our molecular and genetic data indicate that the DAF Smad proteins and DAF-16 FH protein interact on metabolic control promoters.

Interestingly, analogously to *daf-16* bypass of the need for DAF-2 insulin receptor signaling in *daf-16* mutant animals, *lin-31* mutations suppress the need for LET-23 EGF signaling in *C. elegans* vulval development. These findings indicate that the DAF-2 receptor, a downstream signaling molecule (AGE-1), and a transcription factor target DAF-16 are involved in insulin-like signaling in *C. elegans* development. Without being bound by any particular theory, we hypothesize that *C. elegans* insulin signaling via DAF-2 and AGE-1 activate DAF-16 transcriptional activity, so that in a *daf-2* or *age-1* mutant, or in dauer pheromone, DAF-16 acts as a repressor protein causing a metabolic shift to fat metabolism. Our analysis of *daf-16* expression shows that, like DAF-3, it is expressed in target tissues (Fig. 22). Our evidence indicates that Smad protein transcription factors (e.g., DAF 3, DAF8, DAF14) and DAF-16 act on a common set of promoters as combinatorial transcriptional regulators. Thus, it is at these metabolic genes that DAF-7 and TGF- $\beta$ -like and DAF-2 insulin-like signals converge to control metabolism. In addition, our evidence indicates that in the presence of DAF-2 signaling (mimicking high insulin), DAF-16 acts as an activator of transcription, causing a shift in metabolism toward glucose utilization

for cell growth. The molecular analysis described herein suggests that lack of *daf-16* gene activity completely bypasses the need for insulin signaling in metabolic control by releasing metabolic control from DAF-16 repression. These data suggest that if a human DAF-16 homolog acts downstream of insulin signaling in humans, drugs could be developed that inhibit its activity to bypass the need for insulin signaling. Identification of a such a drug should provide a means for treating both Type I and Type II diabetes.

As shown in Figs. 21A-21B, the human FKHR, FKHL1, and AFX genes, identified as oncogene breakpoints but not as insulin signaling genes, are much more closely related to DAF-16 than the next closest relative in either Genbank or in the 94% complete *C. elegans* genome sequence. These data indicate that FKHR, FKHL1, and AFX are excellent candidates for subserving the same function as *C. elegans* DAF-16: transduction of insulin signals and convergence with DAF-7-like Smad signals.

**Evidence for the *C. elegans* AKT kinase as the probable output of DAF-2/AGE-1 signaling**

We screened genetically for mutations that bypass the need for age-1 signaling. This was done by mutagenizing a strain carrying an age-1(mg44) null mutation (this mutation was heterozygous to allow the strain to grow). After two generations, animals that could survive without age-1 gene activity were selected by their lack of arrest at the dauer stage. We identified *daf-16* mutations, as expected. However, we also identified two new gain of function mutations, *sup(mg142)* and *sup(mg144)*.

*sup(mg144)* suppresses three different age-1 alleles, indicating that this

mutation bypasses the need for AGE-1 production of PIP3. For example, *sup(mg144)* suppresses the dauer arrest of *age-1(mg44)*, (*m333*), (*mg109*) such that fertile adults are formed. *sup(mg144)* does not suppress the lack of insulin signaling in the *daf-2* mutant: *daf-2(e1370); sup(mg144)* form dauers at 25 degrees. This suggests that not all of the DAF-2 signaling output is via AGE-1. However, in the absence of both DAF-2 and AGE-1 signaling, *sup(mg144)* weakly suppresses, allowing some fertile adults to bypass arrest at the dauer stage. *daf-2(e1370); sqt-1 age-1(mg44); sup(mg144)* form 8% fertile adults, 12% sterile adults, and 80% dauers at 25 degrees.

Interestingly, *sup(mg144)* is a dominant suppressor of *age-1* mutations. *sqt-1 age-1(mg44); sup(mg144)/+* form 100% fertile adults. The *sup(mg144)* parental genotype does not affect this outcome. This data indicates that *sup(mg144)* is a dominant activating or dominant inactivating mutation.

Genetic mapping indicates that *sup(mg144)* may identify an activating mutation in the *C. elegans* AKT homologue (Fig. 25). By placing *sup(mg144)* in trans to a multiply marked chromosome (using PCR based RFLPs), we found that *sup(mg144)* maps to a 2 map unit genetic interval that includes *C. elegans* AKT (Fig. 24).

In particular, 2/39 *sup(mg144)* homozygous animals isolated from a *sup(mg144)*/polymorphic Bergerac chromosome parent recombined between *sup(mg144)mg144* and *stP6* (these animals also carried *stP18*). In this experiment *mg144* was a heterozygote with RW7000 for three generations, thus placing *sup(mg144)* approximately 2.2mu to the left of *stP6*.

In addition, 1/39 *sup(mg144)* homozygous animals isolated from a *sup(mg144)*/polymorphic Bergerac chromosome parent recombined between

sup(mg144) and bP1. In this experiment mg144 was a heterozygote with RW7000 for two generations. Accordingly, this number is approximately 1/80 or 1.2 mu from bP1.

We generated a GFP fusion to AKT and showed that this gene is  
5 expressed at high levels in dauer larvae but at much lower levels and in fewer cells in wild type animals. (Figs. 26A-26B) Thus AKT represents a dauer regulated gene that may respond to DAF-16 and DAF-3 transcriptional control. Multiple probable binding sites, related to the DAF-3 binding site in myoII have been identified.

10 **sup(mg142) identifies another likely output of age-1 signaling**

mg142 suppresses three different age-1 alleles (age-1(mg44), age-1(m333), and age-1(mg109) at 20 degrees. age-1(mg44); sup(mg142) form fertile adults at 15 and 20 degrees. At 25 degrees, they form 33% fertile adults and 67% sterile adults.

15 sqt-1 age-1(mg44); mg142/+ form 14% fertile adults and 86% sterile adults when the parent was homozygous for mg142. sqt-1 age-1(mg44); mg142/+ form 67% fertile adults and 33% sterile adults when the parent was heterozygous for mg142. daf-2(e1370); mg142 form sterile adults at 25 degrees; daf-2(e1370); sqt-1 age-1(mg44); mg142 form sterile adults and dauers at 25 degrees.

20 Preliminary mapping places mg142 approximately 1.6mu to the left of unc-1 on LGX.

**Novel C. elegans insulin-like hormones are probable DAF-2 ligands**

Mutations in daf-2 not only cause a metabolic shift, but also affect

longevity of *C. elegans*. The nearly complete *C. elegans* genome sequence allowed a definitive search for insulin superfamily members to be performed, and, in this search, we detected multiple insulin-related proteins in the *C. elegans* genome database. When insulin, IGF-I, or IGF-II were compared to the translated worm genome sequence, this large set of insulin superfamily members was not detected. However, when the search was carried out with the conserved signature residues shown below that are the hallmark of the insulin superfamily (SEQ ID NOS: 115, 116), as now defined, we detected a number of novel insulin molecules.

#### Conserved Insulin Motifs

10 1 LCGXXLVEALXXVCGXRGFFYTPKTRRKR GIVEQCCXXXCXXXQL  
EXYCN 50 (SEQ ID NO: 115); and

1 aanqrLCGRHLADALYFVCGNRGFFyspkgGIVEECCHNPCTLYQLE  
NYCn 51 (an insulin superfamily consensus from the Blocks database at  
www.blocks.fhcrc.org; SEQ ID NO: 116).

15 The insulin superfamily signature residues were assembled using a set of  
vertebrate insulins and IGF-I and II proteins as well as silk moth bombyxin (a  
distant insulin relative) and a *Limulus* insulin superfamily member. The use of  
superfamily signature amino acid positions to detect distant relatives in databases  
is a more definitive approach to ascertaining gene superfamily members than  
20 simple searches with single family members.

Using these motifs, eight novel *C. elegans* insulin superfamily members were identified (SEQ ID NOS: 117-124), the coding sequences of which are

shown in Figure 28. In this Figure, the family members are named from the cosmid genomic DNA sequences from which they were detected. All of these insulins have A and B peptide homology to the insulin superfamily, and some of them have conserved dibasic processing sites that would mediate processing of the intervening unconserved C peptide. These genes are widely distributed on the *C. elegans* genome, although some are clustered (for example, ZK75.1, ZK75.2, ZK75.3, and ZK84.6). More distant insulin relatives may exist, but these are likely to engage receptors other than DAF-2.

Of the isolated insulin superfamily members, F13B12 was most closely related to human insulin and IGF-I, II. This was especially obvious from a PILEUP analysis in which a phylogenetic tree of protein superfamily members was constructed (Figs. 29 and 30). The insulin product of F13B12 clustered more closely to the mammalian insulin and IGF-I,II proteins than to other distant relatives like relaxin. Relaxin defined the most distantly related insulin superfamily member in the analysis, and it appeared to engage a tyrosine kinase receptor distinct from the insulin receptor.

These insulin-like hormones are expected to subserve the longevity, dauer arrest, and/or metabolic effects of DAF-2 signaling. For example, each of these insulin superfamily members are expected to engage the DAF-2 receptor, leading to a result in which a mutation in *daf-2* "sums" the functions of these eight or more insulin-like signals.

An analysis of the F13B12 insulin-like hormone is consistent with this view (Tables II-VI). First, as shown below, increasing the dose of the F13B12 insulin-like hormone potently modulates dauer arrest, both in animals carrying weak *daf-2* or weak *daf-7* mutations, and in animals carrying defects in synaptic

components likely to mediate insulin release in *C. elegans* (unc-64).

Table II. High copy F13B12(ins) enhances the Daf-c phenotype of *daf-2(e1365)* at 20°C

5	Parental Genotype	Phenotype of progeny (%)					
		transgenic			non-transgenic		
		dauer	non-dauer	N	dauer	non-dauer	N
10	F13B12 transgenic: <i>daf-2(e1365); mgex309</i>	89.0	11.0	163	2.3	97.7	213
	<i>daf-2(e1365); mgex310</i>	90.5	9.5	220	2.6	97.4	115
	Control transgenic: <i>daf-2(e1365); mgex315</i>	1.8	98.2	283	0.5	99.5	184

Table III. High copy F13B12(ins) maternally suppresses the Daf-c phenotype of *daf-7(e1372)* at 25°C

5	Parental Genotype	Phenotype of progeny (%)					
		transgenic			non-transgenic (but parent was)		
		dauer	non-dauer	N	dauer	non-dauer	N
10	F13B12 transgenic: <i>daf-7(e1372); mgex299</i>	31.4	68.6	236	2.9	97.1	172
	<i>daf-7(e1372); mgex301</i>	16.8	83.2	250	0	100	122
	Control transgenic: <i>daf-7(e1372); mgex312</i>	100	0	78	100	0	60

Table IV. High copy F13B12(ins) maternally suppresses the Daf-c phenotype of *daf-7(e1372)* at 15°C

15	Parental Genotype	Phenotype of progeny (%)					
		transgenic			non-transgenic (but parent was)		
		dauer	non-dauer	N	dauer	non-dauer	N
20	F13B12 transgenic: <i>daf-7(e1372); mgex299</i>	1.4	98.6	73	0.3	99.7	343
	<i>daf-7(e1372); mgex301</i>	0.5	99.5	194	0	100	278
	Control transgenic: <i>daf-7(e1372); mgex312</i>	26.4	73.6	91	25.6	74.4	39

Table V. High copy F13B12(ins) promotes recovery of *unc-64(e246)* dauers at 27°C

Parental Genotype		Phenotype of progeny (%)						
5	Day 2	Day 3						N
		Dauer	Non-dauer	Transgenic Dauer	Transgenic Non-dauer	Non-transgenic Dauer	Non-transgenic Non-dauer	
10	F13B12(ins) transgenic: <i>unc-64(e246); mgex299</i>	91.0	9.0	10.4	56.6	23.6	9.4	106
	<i>unc-64(e246); mgex301</i>	75.3	24.7	22.9	51.1	18.7	7.3	96
	Control transgenic: <i>unc-64(e246); mgex312</i>	88.9	11.1	54.3	10.6	29.8	5.3	208

Table VI. High copy F13B12(ins) enhances the Daf-c phenotype of *unc-64(e246)* at 15°C

20	Parental Genotype	Phenotype of progeny (%)					
		transgenic			non-transgenic		
		dauer	non-dauer	N	dauer	non-dauer	N
	F13B12 transgenic: <i>unc-64(e246); mgex299</i>	23.2	76.8	185	0	100	170
	<i>unc-64(e246); mgex301</i>	36.0	64.0	75	0	100	77
	Control transgenic: <i>unc-64(e246); mgex312</i>	0	100	177	0	100	134

A genetic analysis has shown that high F13B12 insulin-like hormone signaling can suppress dauer arrest induced by *daf-7* mutations or decreases in synaptic signaling, but can enhance dauer arrest caused by decreases in *daf-2* signaling. Thus, the F13B12 insulin-like hormone may act synergistically with DAF-7 signals, like the DAF-2 receptor, but may interfere with the secretion or activity of another DAF-2 ligand. These genetic data strongly implicate the F13B12 insulin-like hormone in DAF-2 signaling.

In addition, the expression pattern of a promoter fusion of the F13B12 insulin-like hormone to GFP is also consistent with the genetic results. In these experiments, GFP was expressed in several head neurons, including ASJ and ASH, a pair of pharyngeal neurons, with processes that looked most like NSM, and three tail neurons. The full-length GFP looked similar but very faint. Worms expressing the full-length GFP lived longer than wild type. Interestingly, the NSM neuron had dense core vesicles by EM analysis, which is also true of beta cells of the pancreas. Pancreatic beta cells are also neuronal in character; they use synaptic components for insulin vesicle release, are synaptically connected to the autonomic nervous system, and are electrically active. Sulfonyl ureas, which are used to increase insulin release, act by regulating the activity of K channels in beta cells, much the way K channels regulate excitability in other neurons. Finally, the NSM neuron is a part of the *C. elegans* enteric nervous system, just like the pancreas in mammals. Accordingly, the expression and functional analysis of the F13B12 insulin-like hormone is highly supportive of its role in insulin-like control of worm metabolism and aging.

Although the F13B12 insulin-like hormone is the closest *C. elegans* homologue to insulin, it is likely that many or all of these insulin superfamily

members engage the DAF-2 receptor to regulate their activity. For example, they are more closely related to insulin than to the ligands of the other growth factor receptors present in the worm genome. These distinct insulin superfamily ligands could regulate DAF-2 at distinct times or places, or act antagonistically or synergistically to the F13B12 insulin-like hormone. Some of these insulin-like hormones may regulate metabolism, like insulin, whereas others may regulate dauer arrest or longevity. Thus, the *daf-2* mutant phenotype that results from loss of the receptor for these many hormones may be a composite loss of many hormonal signals. Consistent with such a model, neuronal expression of the DAF-2 receptor in a *daf-2* null mutant has been found to complement the dauer arrest phenotype of a *daf-2* mutant but not the metabolic or aging defects. Accordingly, one DAF-2 ligand may be expressed in or near the brain to control dauer arrest, but other ligands may impinge on DAF-2, for example, in non-neuronal cells, to control metabolism and aging.

By this view, loss of only one of the insulin-like hormones may cause only a subset of the *daf-2* mutant phenotype, for example, only increased longevity or only metabolic dysregulation. These *C. elegans* insulin superfamily members may, for example, subserve the longevity or senescence function of DAF-2 receptor signaling, and an increase in such a hormone activity late in life may actually mediate the increase in DAF-2 activity that causes senescence. Conversely, if any of these insulin-like proteins have antagonistic effects on DAF-2, any decline in their activity late in life could mediate senescence. Application of only one hormone by injection or germ line therapy could therefore be used to target, for example, aging without any effects on metabolism.

In addition, since the F13B12 insulin-like hormone is a detectable worm

homologue of insulin, it is possible that the other 7 worm insulins also have human homologues that are more closely related to their nematode counterparts than they are to each other. In fact the divergence of the F13B12 insulin-like hormone from insulin and IGF-I and IGF-II gives a measure of how much divergence may be expected for the mammalian homologues of the other insulin superfamily members. The F13B12 insulin-like hormone is slightly more closely related to IGF-II than insulin or IGF-I, but these three genes are probably duplicated and diverged homologues of a F13B12 homologue in the common ancestor of *C. elegans* and *Homo sapiens*. In fact, it is a current rule of thumb that many gene families in mammals have 4 times as many members as in *C. elegans*. For example, there are 4 Hox clusters in mammals and only one in *C. elegans*. Similarly, there are 3 known DAF-2 receptor homologues and DAF-16 transcription factor homologues in mammals (it is likely that the fourth mammalian member of these gene families will become known when the full mammalian genome sequence is finished). Thus, it is reasonable to expect that, for every insulin like protein in *C. elegans*, there may be four in mammals, or a total of 24 for the family of 8 shown above. In addition, since the F13B12 insulin-like hormone is expressed in only a few neurons, it is possible that the other insulin superfamily members are similarly expressed in a small set of neurons, and that the human homologues may be expressed in only rare regulatory cell types.

The insulin-like hormones described herein, as well as their human homologues, provide valuable candidate regulators of senescence. For example, if human senescence is triggered by a decline in an insulin-like longevity hormone, in analogy to how puberty is triggered by a timed change in sexual maturation hormones, it may prove possible to regulate the aging process in the same way that

sexual maturation can be regulated by hormone treatment. In addition, the *C. elegans* aging hormones may reveal which human genes have such a function. Because *daf-2* mutations cause longevity increases in a manner analogous to caloric restriction in mammals, it is possible that caloric restriction in mammals regulates the level of an insulin-like hormone that in turn engages the insulin or IGF-I, II receptors. Such a hormone may not have been detected if its level is very low or if it signals over a short range. However, once the human genome sequence is complete, the detection of human homologues to the *C. elegans* superfamily members listed above will become a trivial matter of database searching. In this way, the determination of the function of the worm homologue function in longevity or growth arrest or metabolism control will supply valuable functional information about the activity of human homologues.

The effect of the *C. elegans* insulin-like proteins on longevity, metabolism, or growth arrest may be readily determined by a combination of high copy studies, as shown above for the F13B12 insulin-like hormone, as well as by using RNA inhibition and knockout strategies to inhibit the activities of these genes. The *C. elegans* strains are then tested for interactions with *daf* pathway mutants, for example, as shown for the F13B12 insulin-like hormone above, and for longevity effects by standard techniques.

The human proteins that regulate longevity may be detected by a combination of database searches and genetic complementation of worm RNAi or gene knockout mutants (for example, as described herein), as well as by high copy effects of human genes on worm longevity and metabolic control.

Because these human proteins are hormones, they may be used to directly regulate human longevity, for example, by injection into the bloodstream.

Depending on the particular hormone and its effects, the hormones themselves may cause increased longevity, or they may be modified to generate dominant interfering hormones (for example, by engineering chimeras between the insulin superfamily members). The function of these proteins upon injection into the  
5 bloodstream may be predicted from their function in *C. elegans*, for example, as ascertained by transgenic analysis. Because of their effects on longevity, the human homologues of these *C. elegans* insulin-like endocrine signals have important applications in preventing or retarding the aging process.

***C. elegans Akt/PKB Transduces Insulin Receptor-like Signals from AGE-1***

10 ***Phosphoinositide-3-OH kinase to the DAF-16 Transcription Factor***

An insulin receptor-like signaling pathway regulates *C. elegans* metabolism, development, and longevity (Kimura et al., *Science* 277:942-946, 1997). In response to a secreted pheromone, wild type animals arrest development at the dauer stage with a concomitant switch to fat storage metabolism in the  
15 intestine and hypodermis, increased lifespan, and remodelling of many tissues (Kimura et al., *Science* 277:942-946, 1997; Riddle and Albert, in *C. elegans II*, eds. Riddle, D.L., Blumenthal, T., Meyer, B.J. & Priess, J.R., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 739-768, 1997). Mutations in the insulin/IGF-I receptor homolog *daf-2* (Kimura et al., *Science* 277:942-946, 1997)  
20 or in the phosphoinositide-3-OH kinase (PI3K) homolog *age-1* (Morris et al., *Nature* 382:536-539, 1996) cause constitutive arrest at the dauer stage; genetic analysis is consistent with AGE-1 functioning downstream of DAF-2 (Gottlieb and Ruvkun, *Genetics* 137:107-120, 1994; Larsen et al., *Genetics* 139:1567-1583, 1995). Mutations in the Fork head transcription factor DAF-16 completely

suppress the dauer arrest, metabolic shift, and longevity phenotypes of *daf-2* and *age-1* mutants (Gottlieb and Ruvkun, *Genetics* 137:107-120, 1994; Larsen et al., *Genetics* 139:1567-1583, 1995; Kenyon et al., *Nature* 366:461-464, 1993; Ogg et al., *Nature* 389:994-999, 1997; Lin et al., *Science* 278:1319-1322, 1997),

5 indicating that DAF-16 is a negatively regulated downstream target of *C. elegans* insulin receptor signaling. Molecules that couple the DAF-2 insulin receptor protein and AGE-1 PI3K to the DAF-16 transcription factor have not been identified by previous extensive genetic screens. While biochemical studies have suggested that the mammalian Akt/PKB (also known as RAC) serine/threonine  
10 kinase may transduce signals from PI3Ks associated with receptor tyrosine kinases (Franke et al., *Cell* 81:727-736, 1995; Burgering and Coffey, *Nature* 376:599-602, 1995; Cross et al., *Nature* 378:785-589, 1995), such as the insulin receptor to downstream effectors, this has not been demonstrated by genetic analysis of signaling pathways in whole organisms. We established the action of *C. elegans*  
15 Akt/PKB in the DAF-2 insulin receptor-like signaling pathway by the genetic identification of an activating Akt/PKB mutation and by genetic analysis of Akt/PKB inactivation and overexpression.

An activating mutation (*mg144*) in *akt-1*, one of two *C. elegans* Akt/PKB homologs, was identified in a genetic screen for mutations that suppress  
20 the dauer arrest phenotype of the *age-1(mg44)* null mutant (Morris et al., *Nature* 382:536-539, 1996). This screen was designed to isolate reduction of function mutations in molecules negatively regulated by PI3K signaling, or gain of function mutations in molecules positively regulated by PI3K signaling. Among 10 independent suppressor mutations isolated in a screen of 3800 haploid genomes, in  
25 addition to the activating *akt-1* mutation, we also isolated multiple alleles of a

previously known negatively regulated target, *daf-16* (Gottlieb and Ruvkun, *Genetics* 137:107-120, 1994; Larsen et al., *Genetics* 139:1567-1583, 1995) and one other suppressor that maps to the *daf-16* interval between *lin-11* and *unc-75*, suggesting that the screen revealed genes that act in this insulin-like signaling pathway. Another dominant mutation, *mg142*, that suppresses multiple *age-1* alleles and six mutations that vary in their ability to suppress multiple *age-1* alleles were also isolated in the screen.

The *mg144* mutation suppresses the three *age-1* alleles tested, including two classes of nonsense alleles and one missense substitution (Ala845Thr) in a conserved region of PI3K (Morris et al., *Nature* 382:536-539, 1996). *mg144* is completely dominant for suppression of the dauer constitutive phenotype of *age-1(mg44)* (75.1% of the progeny of *age-1(mg44); mg144/+* animals developed as non-dauers, and 24.9% arrested at the dauer stage, N=774). On its own, *mg144* does not have any obvious phenotypes; it moves normally, has a normal vulva and brood size, and makes dauers on starved plates and on plates treated with pheromone. Thus *mg144* does not activate the AGE-1 PI3K signaling pathway to the point that normal dauer arrest is affected but does activate the pathway sufficiently to alleviate the requirement for AGE-1 PI3K outputs.

Using suppression of the dauer constitutive phenotype of *age-1(mg44)*, *mg144* was mapped to a region on chromosome V within 1.3mu of the polymorphic STS marker bP1 (Fig. 31). From the *C. elegans* genome sequence in this 1.3 mu region, we identified a *C. elegans* Akt/PKB homolog which we named *akt-1* (Fig. 31). Because an activating mutation in Akt/PKB is a good candidate to be a genetically dominant suppressor of an *age-1* PI3K null mutant, we determined the *akt-1* DNA sequence in the *mg144* strain by PCR amplification and direct

sequencing. The *akt-1* gene in the *mg144* mutant strain was shown to bear an Ala183Thr substitution (Fig. 34). *akt-1* is differentially spliced within the conserved kinase domain to generate the *akt-1a* and *akt-1b* isoforms with distinct kinase domain subregions IV, V, and VI (92% identical, 238/258 amino acids over the entire kinase domain; 69% identical, 44/64 amino acids in the differentially spliced region). *akt-1a* is 58% identical to human Akt/PKBa (Fig. 33 and 34). *akt-1* has a pleckstrin homology domain, kinase domain, and the two phosphorylation sites necessary for Akt/PKB activation (Alessi et al., *EMBO J.* 15:6541-6551, 1996) which are the hallmarks of the Akt/PKB family (Fig. 34).

The next most closely related non-Akt/PKB mammalian kinase is rat PKC $\beta$ 1 which is 38% identical to *akt-1a*. The *akt-1(mg144)* mutation is present in both splice forms of *akt-1* and is located in a region of the protein that links the N-terminal pleckstrin homology domain to the C-terminal kinase domain. This mutation is in a region that is not conserved between *C. elegans* and mammalian Akt/PKB. This mutation may reveal a negative regulatory region on *akt-1* because the *mg144* allele is an activating mutation (see below).

To confirm that the *mg144* suppression of *age-1* that is genetically linked to *akt-1* was due to a mutation in *akt-1*, we used a reverse genetic assay termed RNA interference (RNAi) (Fire et al., *Nature* 391:806-811, 1998; Rocheleau et al., *Cell* 90:707-716, 1997; Zhang et al., *Nature* 390:477-484, 1997) to decrease *akt-1* gene activity in an *age-1(mg44); akt-1(mg144)* strain. If a mutation in *akt-1* was responsible for the suppression of *age-1* observed in this strain, RNAi of *akt-1* in this strain should revert the suppression phenotype and result in a dauer constitutive phenotype. This experiment was conceptually similar to the classic genetic arguments that show that a cis-acting loss of function

mutation can revert a gain of function mutation in the same gene. Inhibition of *akt-1* activity in an *age-1(mg44); akt-1(mg144)* strain reverted the *akt-1(mg144)* suppression phenotype, indicating that the *mg144* activating mutation was a lesion in the *akt-1* locus.

5           We identified another Akt/PKB homolog in the nearly complete *C. elegans* genome sequence (Wilson et al., *Nature* 368:32-38, 1994) which we named *akt-2* (Fig. 32). *akt-1* and *akt-2* are more closely related to each other (66% identity between *akt-1a* and *akt-2* overall) than to any other Akt/PKB homolog (Fig. 33). *akt-2* is 55% identical to human Akt/PKBa overall and 35% identical to  
10   rat PKC $\beta$ 1 overall. Interestingly, *akt-2* only has the Thr308 phosphorylation site that is necessary for Akt/PKB activation by PDK1 (Alessi et al., *Current Biology* 7:261-269, 1997; Stokoe et al., *Science* 277:567-570, 1997) but not the Ser473 phosphorlyation site (Alessi et al., *EMBO J.* 15:6541-6551, 1996) (Fig. 34) and yet clearly functions in the insulin-like signaling pathway (see below).

15           Reduction of both *akt-1* and *akt-2* activities revealed that they transduce insulin-like signals from the AGE-1 PI3K to the DAF-16 forkhead transcription factor. Inhibition of either *akt-1* or *akt-2* activity by RNAi did not cause dauer arrest. However, simultaneous inhibition of both *akt-1* and *akt-2* activities caused nearly 100% arrest at the dauer stage. We concluded that Akt/PKB signaling from  
20   either *akt-1* or *akt-2* is sufficient for reproductive development. This result indicates that *akt-1* and *akt-2* can function redundantly for dauer formation in *C. elegans* and raises the possibility that various mammalian Akt/PKB isoforms could function redundantly as well. Significantly, the constitutive dauer arrest induced by inhibition of both *akt-1* and *akt-2* is fully suppressed by a null mutation in  
25   *daf-16* (Ogg et al., *Nature* 389:994-999, 1997) but is not suppressed by a null

mutation in the Smad homolog *daf-3* (Patterson et al., *Genes & Development* 11:2679-2690, 1997) which confirms its placement in the DAF-2/AGE-1/DAF-16 signaling pathway. Because a null mutation in *daf-16* alleviates the need for *C. elegans* Akt/PKB signaling, the primary function of AKT-1 and AKT-2 is to antagonize DAF-16. Interestingly, DAF-16 contains four consensus sites for phosphorylation by Akt/PKB (Alessi et al., *FEBS Letters* 399:333-338, 1996) and three of these sites are conserved in the human DAF-16 homologs AFX, FKHR, and FKHL1. AKT-1 and AKT-2 may exert their negative regulatory effect by directly phosphorylating DAF-16. Shown below are comparisons of AFX, FKHR, and DAF-16, indicating the conservation between the consensus phosphorylation sites. The AKT sites indicated are located downstream and upstream, respectively, of the Forkhead domain (SEQ ID NOS: 161-169).

Score = 151 (68.4 bits), Expect = 1.9e-140, Sum P(8) = 1.9e-140

Identities = 28/54 (51%), Positives = 38/54 (70%)

```
AFX:      226 SPVGHFPAKWSGSPCSRNRREEADMWTTFRPRSSSNASSVSTRLSPLRPESEVLAE 279
          SP   F+KW   SP S + ++ D W+TFRPR+SSNAS++S RLSP+   E + L E
FKHR:     287 SPGSQFSKWPASPGSHSNDDFDNWSTFRPRTSSNASTISGRLSPIMTEQDDLGE 340
DAF-16a                                SFRPRTQSNLSIPGSSS
```

Score = 132 (59.8 bits), Expect = 1.9e-140, Sum P(8) = 1.9e-140

Identities = 22/42 (52%), Positives = 28/42 (66%)

```
AFX:          7 KAAAIIDLDPDFEPQSRPRSCTWPLPRPEIANQPSEPPEVEP 48
              +A  ++++DPDFEP  RPRSCTWPLPRPE +   S       P
FKHR:         3 EAPQVVEIDPDFEPLRPRSCTWPLPRPEFSQNSATSSPAP 44
DAF-16      25 TFMNTPDDVMMNDDMEPIPRDRCNTWPMRRPQLEPPLNSSP 177
              T  ++P+ V ++ D EP+PR R  TWP+ RP++  +  +++++
```

We have shown that human AKT will phosphorylate *C. elegans* DAF-16 and that this phosphorylation is dependent on these sites. Upon mutation of the serine or threonine in these sites to alanine, *in vitro* phosphorylation of DAF-16 (or fragments of DAF-16) is abolished. It is expected that the lack of *akt* input to DAF-16 in these mutant nematodes will result in dauer arrest, just like animals lacking *akt-1/akt-2* gene activity.

The above genetic results show that Akt/PKB is the major output of PI3K signaling and implicate a transcription factor downstream target for the Akt/PKB kinase. Because mutations in *daf-16* suppress *akt-1* and *akt-2* reduction of function, it is likely that DAF-16 represents a major signaling output of Akt/PKB in *C. elegans* insulin-like signaling. Akt/PKB has been implicated in mammalian insulin receptor signaling that localizes glucose transporters to the plasma membrane (Kohn et al., *J. Biol. Chem.* 271:31372-31378, 1996) and has been shown to regulate glycogen synthesis via direct phosphorylation of GSK-3 (Cross et al., *Nature* 378:785-589, 1995), two events which are not transcriptionally regulated. While there also may be such Akt/PKB outputs in *C. elegans*, the DAF-16 Fork head transcription factor represents the major output of DAF-2/AGE-1/AKT-1/AKT-2 insulin receptor-like signaling (Ogg et al., *Nature* 389:994-999, 1997). Similarly Akt/PKB action in the insulin/IGF-I anti-apoptotic pathway (Dudek et al., *Science* 275:661-665, 1997; Kauffmann-Zeh et al., *Nature* 385:544-548, 1997; Kulik et al., *Mol. Cell Biol.* 17:1595-1606, 1997 24-26) may also converge on transcription factors related to DAF-16.

The normal requirement of *age-1* activity for reproductive development is also bypassed by increased gene dosage of wild type *akt-1*. Transgenic

*age-1(mg44)* animals carrying a 7.3 kb *akt-1(+)* genomic region can grow reproductively rather than arrest at the dauer stage. Greater than 75% of *age-1(mg44)* animals that contain the *akt-1(+)* transgene at high copy bypass dauer arrest while non-transgenic *age-1(mg44)* animals never bypass dauer arrest. This rescue is dependent on a conserved lysine residue implicated in mammalian AKT/PKB kinase activity (Franke et al., *Cell* 81:727, 1995). In a similar experiment with *age-1(mg44)* animals carrying the same genomic region amplified from *akt-1(mg144)* at high copy, the transgenic animals bypassed dauer arrest at a similar frequency. The *age-1(mg44)* animals carrying the *akt-1(mg144)* transgene at low copy bypass dauer arrest more frequently than the *age-1(mg44)* animals carrying the *akt-1(+)* transgene at low copy (approximately 85% of *age-1(mg44)* animals carrying *akt-1(mg144)* transgene bypass dauer compared to 38% of *age-1(mg44)* animals carrying the *akt-1(+)* transgene). These results indicate that the same 7.3 kb genomic region amplified from the *akt-1(mg144)* strain is a more potent suppressor of *age-1(mg44)* than the *akt-1(+)* transgene. These data map *mg144* to the 7.3 kb region of *akt-1* that includes the Ala183Thr substitution in AKT-1. However, while multiple independent *akt-1(mg144)* transgenes are more potent suppressors of *age-1(mg44)* than *akt-1(+)* transgenes, which suggests that more *akt-1* gene activity is generated by *akt-1(mg144)*, there is significant variation in the penetrance of suppression observed with different transgenes. In addition, even though *akt-1(+)* transgenes confer suppression of *age-1(mg44)* that is not observed with chromosomal *akt-1(+)*, the penetrance of suppression of *age-1(mg44)* by either *akt-1(+)* or *akt-1(mg144)* transgenes is less than from *akt-1(mg144)/+* heterozygotes or *akt-1(mg144)* homozygotes. This may be due to mosaicism of *akt-1* gene expression from transgenic arrays or a saturation of *akt-1*

gene function by high gene dosage. These data also suggest that the mutation may act by increasing AKT-1 abundance or stability, thus conferring the ability to grow in the absence of age-1 signaling.

Null mutations in age-1 cause dauer arrest as does inactivation of *akt-1* and *akt-2* by RNAi. This indicates that *akt-1(+)*, *akt-2(+)*, and *age-1(+)* are required for reproductive development. Because the dominant allele *akt-1(mg144)* also promotes reproductive growth by virtue of its ability to suppress the dauer constitutive phenotype of *age-1* null mutants, it functions similarly to *akt-1(+)* and *akt-2(+)*. Thus *akt-1(mg144)* is an activating mutation, as opposed to a loss of function or dominant negative mutation in *akt-1*. In addition, the fact that both *akt-1(mg144)* and providing additional copies of the *akt-1(+)* gene suppress an *age-1* null mutant is consistent with *akt-1(mg144)* being an activating mutation.

Because *akt-1* and *akt-2* function redundantly to repress dauer formation we asked whether overexpression of *akt-2(+)* could also bypass the normal requirement of AGE-1 PI3K signaling. *age-1(mg44)* animals carrying the *akt-2(+)* transgene arrested as dauers while *age-1(mg44)* animals carrying the *akt-1(+)* transgene bypassed dauer. Thus, either because of differences in the AKT-2 protein or differences in protein expression, high gene dosage of *akt-2* is not able to bypass the usual requirement for AGE-1 PI3K signaling.

*akt-1(mg144)* suppresses the dauer constitutive phenotype of three *age-1* alleles. Because *age-1(mg44)* is a null mutant, these data strongly suggest that *akt-1* acts downstream of *age-1* and demonstrates that the biochemical ordering of PI3K upstream of Akt/PKB kinase is also true in an intact organism. AGE-1 is the only PI3K homolog in *C. elegans* of the type regulated by tyrosine kinase receptors. Significantly, our results demonstrate that *C. elegans* Akt/PKB gene

activity is not strictly dependent on upstream *age-1* activity if Akt/PKB activity is increased because *akt-1(mg144)* as well as *akt-1(+)* overexpression suppress null mutations in AGE-1 PI3K. This is comparable to the suppression by *daf-16(m27)*, a reduction of function allele (Lin et al., *Science* 278:1319-1322, 1997), and *daf-16* null alleles (Ogg et al., *Nature* 389:994-999, 1997).

A mutation in *daf-2* is suppressed more poorly by *akt-1(mg144)* than by a reduction of function mutation in *daf-16*. The *age-1* alleles suppressed by *akt-1(mg144)* are null (Morris et al., *Nature* 382:536-539, 1996) whereas *daf-2(e1370)* is a temperature sensitive mutation in the kinase domain (Kimura et al., *Science* 277:942-946, 1997). This *daf-2* allele is completely suppressed by many *daf-16* alleles, including null alleles (Gottlieb and Ruvkun, *Genetics* 137:107-120, 1994; Larsen et al., *Genetics* 139:1567-1583, 1995; Ogg et al., *Nature* 389:994-999, 1997). This result, in comparison to the robust suppression of *age-1* mutations by *akt-1(mg144)*, suggests that akt-1 is a major output of AGE-1 signaling and one of multiple outputs of DAF-2 signaling. In addition, because *akt-1(mg144)* can bypass the need for AGE-1 PI3K signaling but not for DAF-2 insulin receptor-like signaling, *akt-1(mg144)* defines a bifurcation in the signaling pathway downstream of *daf-2*. It is likely that *age-1* and *akt-1* constitute one major signaling pathway from DAF-2 and that other, as yet unidentified genes, constitute one or more parallel pathways. These pathways converge downstream of AGE-1 and at or upstream of the DAF-16 Fork head transcription factor and negatively regulate its activity, since loss of function mutations in *daf-16* completely suppress both *daf-2* and *age-1* mutations (Gottlieb and Ruvkun, *Genetics* 137:107-120, 1994). Because a decline in AGE-1 PI3K or AKT-1/AKT-2 signaling induces dauer arrest in the presence of signaling from

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this parallel pathway, both are necessary for reproductive development. The genetic evidence for multiple DAF-2 insulin receptor-like outputs demonstrate that biochemical studies showing that parallel PI3K, ras, SHP2, and other signaling outputs are activated by the insulin receptor in mammals (Kahn, *Diabetes* 5 43:1066-1084, 1994) are relevant to insulin receptor-like signaling in intact organisms.

In addition, a mutation in *daf-2* is suppressed more poorly by *akt-1(mg144)* than by a reduction of function mutation in *daf-16*. The *age-1* alleles suppressed by *akt-1(mg144)* are null (Morris et al. (1996) *Nature* 382:536-10 539) whereas *daf-2(e1370)* is a temperature sensitive mutation in the kinase domain (Kimura et al. (1997) *Science* 277:942-946). This *daf-2* allele is completely suppressed by many *daf-16* alleles, including null alleles (Gottlieb and Ruvkun (1994) *Genetics* 137:107-120; Larsen et al. (1995) *Genetics* 139:1567-1583; Ogg et al. (1997) *Nature* 389:994-999). This result, in comparison to the 15 robust suppression of *age-1* mutations by *akt-1(mg144)*, suggests that AKT-1 is a major output of AGE-1 signaling and one of multiple outputs of DAF-2 signaling.

Overexpression of either *akt-1(+)* or *akt-1(mg144)* can bypass the need for DAF-2 signaling while overexpression of *akt-2(+)* or *akt-1(KD)* does not alleviate the need for DAF-2 signaling. However, *akt-1(+)* and *akt-1(mg144)* 20 transgenes are more efficient suppressors of the dauer constitutive phenotype of *age-1(mg44)* than of *daf-2(e1370)*. This supports the model that AKT-1 is a primary output of AGE-1 signaling but not DAF-2 signaling.

Reduction of zygotic *age-1* activity increases *C. elegans* lifespan greater than two-fold (Morris et al., *Nature* 382:536-539, 1996; Larsen et al., *Genetics* 25 139:1567-1583, 1995; Klass, *Mech. Ageing Dev.* 22:279-286, 1983). Mutations in

*daf-16* suppress this lifespan increase (Larsen et al., *Genetics* 139:1567-1583, 1995; Dorman et al., *Genetics* 141:1399-1406, 1995). *akt-1(mg144)* does not suppress the *age-1(mg44)* induced increase in lifespan (for the following strains, mean lifespans, maximum lifespan are given: N2 12 days, 16 days, N=28; *sqt-1(sc13) age-1(mg44)* 18 days, 36 days, N=20; *sqt-1(sc13) age-1(mg44); akt-1(mg144)* 22 days, 38 days, N=36; *daf-16(m27); sqt-1 (sc13) age-1(mg44)* 14 days, 16 days, N=32). Thus *akt-1(mg144)* bypasses the need for AGE-1 signaling in reproductive development but does not activate normal aging pathways. It is possible that *akt-1(mg144)* does not subserve all the functions of the wild type *akt-1* or *akt-2*. *akt-2* or other as yet unidentified downstream effectors of *age-1* may be the pertinent signaling molecules for lifespan regulation.

The expression patterns of both *akt-1* and *akt-2* were examined in transgenic animals containing a translational fusion of each genomic locus to Green Fluorescent Protein (GFP) (Chalfie et al., *Science* 263:802-805, 1994). The GFP fusion proteins contain the entire genomic coding region from either *akt-1* or *akt-2*, including 5' upstream regulatory sequence, fused in frame at the C-terminus to GFP. The AKT-1/GFP construct is sufficient to suppress the dauer constitutive phenotype of *age-1(mg44)* while the AKT-2/GFP construct is not. This result is not unexpected because increased gene dosage of *akt-2(+)* does not suppress *age-1(mg44)* while increased gene dosage of *akt-1(+)* does. AKT-1/GFP expression is first observed in late embryos and is maintained throughout the life of the animal. In post-embryonic animals, AKT-1/GFP is expressed in the majority of head neurons including sensory neurons. Expression is also observed in motor neurons of the ventral and dorsal nerve cord, neuronal commissures and processes throughout the body, and the tail neurons. The fusion protein is

localized throughout the cell body and axonal and dendritic processes of neurons but is usually excluded from the nucleus. Additional tissues which consistently express AKT-1/GFP include neurons and muscle cells of the pharynx, the rectal gland cells, and the spermatheca. AKT-1/GFP expression was observed more  
5 variably in a variety of cell types including hypodermis, intestine, muscle, some of the P cell descendants that form the vulva, and in the excretory canal.

Consistent with redundant roles of *akt-1* and *akt-2*, an AKT-2/GFP full length protein fusion gene is expressed at the same times as AKT-1/GFP and in the same tissues that express AKT-1/GFP, although AKT-2/GFP seems to be less  
10 abundant. In dauers induced by starvation on crowded plates, AKT-1/GFP and AKT-2/GFP expression does not differ dramatically from their expression during reproductive growth. These expression patterns are consistent with AKT-1 and AKT-2 functioning either in secretory neurons to regulate dauer arrest and metabolic shift or in the target tissues that are remodeled during dauer formation  
15 such as the pharynx, hypodermis, and intestine.

The activating mutation *akt-1(mg144)*, as well as overexpression of *akt-1(+)*, bypasses the normal requirement for AGE-1 PI3K signaling in the DAF-2 insulin receptor-like signal transduction pathway. These results demonstrate that *C. elegans* Akt/PKB gene activity is not strictly dependent on  
20 upstream *age-1* activity if Akt/PKB activity is increased. In the almost complete *C. elegans* genome sequence, AGE-1 is the only PI3K homolog of the type known to generate 3-phosphoinositides. If AGE-1 is the only protein able to generate 3-phosphoinositides in *C. elegans*, these results suggest that, while normal AKT-1 signaling is dependent on 3-phosphoinositides, AKT-1 can become activated in  
25 their absence if gene dosage is increased or the *mg144* mutation is introduced.

Importantly, either activated *akt-1* or higher *akt-1*(+) gene dosage does not efficiently suppress mutations in the DAF-2 insulin receptor suggesting that *age-1* and *akt-1* constitute one major signaling pathway from DAF-2 and that other, as yet unidentified genes, constitute one or more parallel pathways. These pathways most likely converge on the DAF-16 Fork head transcription factor and negatively regulate its activity, since loss of function mutations in *daf-16* completely suppress both *daf-2* and *age-1* mutations (Gottlieb and Ruvkun (1994) Genetics 137:107-120; Larsen et al. (1995) Genetics 139:1567-1583), as well as inactivation of *akt-1* and *akt-2* signaling.

While AKT-1 and AKT-2 appear to function redundantly in transduction of DAF-2/AGE-1 signals, increased *akt-1* gene dosage is a much more potent suppressor of *age-1* null mutations than increased *akt-2* gene dosage. A major distinction between AKT-1 and AKT-2 is that AKT-1 bears two distinct phosphorylation sites (corresponding to Thr308 and Ser473 in human Akt/PKBa) that are necessary for activation of Akt/PKB by upstream growth factor inputs (Alessi et al. (1996) EMBO J. 15:6541-6551; Alessi et al. (1996) FEBS Letters 399:333-338) while AKT-2 only has the Thr308 phosphorylation site. In mammals, Akt/PKB is phosphorylated at Thr308 by PDK1 and at Ser473 by the as yet unpurified PDK2 (Alessi et al. (1997) Current Biology 7:261-269; Stokoe et al. (1997) Science 277:567-570). Thus AKT-1 may couple to a PDK2-like kinase whereas AKT-2 cannot do so. AKT-1 and AKT-2 may also differ in other kinase inputs or in their substrates. Interestingly, at lower temperatures, the *akt-2*(+) transgene can supply sufficient Akt/PKB activity to weakly suppress the dauer arrest caused by *age-1(mg44)*. Temperature is a major modulator of dauer arrest (Riddle and Albert (1997) Genetic and Environmental Regulation of Dauer Larva

Development. In *C. elegans* II (ed. D.L. Riddle, T. Blumenthal, B.J. Meyer and J.R. Priess), pp. 739-768, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The penetrance of dauer arrest in most dauer constitutive mutants is increased at high temperatures (Riddle and Albert (1997) Genetic and

5 Environmental Regulation of Dauer Larva Development. In *C. elegans* II (ed. D.L. Riddle, T. Blumenthal, B.J. Meyer and J.R. Priess), pp. 739-768, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), suggesting that some signals in the pathway are enhanced at low temperature. Thus at low temperatures perhaps PDK1 signaling to AKT-1 and AKT-2 or signaling in pathways parallel to

10 AGE-1/AKT-1/AKT-2 are enhanced, allowing increased *akt-2(+)* gene dosage to weakly bypass the normal requirement for AGE-1 PI3K signaling.

Insulin-like and TGF- $\beta$  neuroendocrine signals regulate whether animals arrest at the dauer stage or grow to reproductive adults (Kimura et al. (1997) Science 277:942-946; Riddle and Albert (1997) Genetic and Environmental

15 Regulation of Dauer Larva Development. In *C. elegans* II (ed. D.L. Riddle, T. Blumenthal, B.J. Meyer and J.R. Priess), pp. 739-768, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The TGF- $\beta$ -like molecule DAF-7 is a probable neuroendocrine signal: it is expressed in the sensory neuron ASI that represses dauer arrest (Bargmann and Horvitz (1991) Science 251:1243-1246) and

20 its expression is regulated by dauer-inducing pheromone (Ren et al. (1996) Science 274:1389-1392; Schackwitz et al. (1996) Neuron 17:719-728). While the insulin-like ligand for the DAF-2 insulin-like receptor has not yet been identified, it may also be produced by secretory neurons and regulated by pheromone. Precedent from biochemical analysis predicts that DAF-2, AGE-1, AKT-1/AKT-2,

25 and DAF-16 function in the same cells. It is not yet clear whether the DAF-2

signaling pathway acts in the target tissues that are remodeled in dauer larvae such as the pharynx, hypodermis, and intestine, or in other signaling cells that in turn control target tissues. The broad expression pattern of *akt-1* and *akt-2* includes the nervous system, pharynx, and hypodermis. This expression pattern is consistent with a role for these genes either in sensory neurons that signal to repress dauer arrest or in the target tissues that receive the dauer repressing signal. The expression patterns of *daf-2* and *age-1* have not been reported; *daf-16* is widely expressed (Ogg et al. (1997) Nature 389:994-999) as are *daf-3* and *daf-4*, two genes that comprise the DAF-7 TGF- $\beta$  signal reception pathway (Patterson et al. (1997) Genes and Development 11:2679-2690). Mosaic or tissue-specific expression analysis will be required to demonstrate in which cell types the DAF-2 insulin-like and DAF-1/DAF-4 TGF- $\beta$  signal transduction pathways act.

The role of AKT-1 and AKT-2 in regulating the metabolic shift and developmental arrest associated with dauer formation suggests the following model. Under normal growth conditions, an insulin-like molecule binds to the DAF-2 insulin receptor kinase inducing autophosphorylation and recruitment of AGE-1 PI3K. As discussed herein, PI3K signals via Akt/PKB. Precedent from biochemical experiments in other systems (Franke et al., *Cell* 81:727-736, 1995; Franke et al., *Science* 275:665-668, 1997; Klippel et al., *Mol. Cell Biol.* 17:338-344, 1997) suggests that AGE-1 activation produces phospholipids that bind to and activate AKT-1 and AKT-2 by inducing a conformational change in the protein that makes it accessible to phosphorylation events which are necessary for activation (Alessi et al., *Current Biology* 7:261-269, 1997; Stokoe et al., *Science* 277:567-570, 1997). A parallel pathway or pathways from the DAF-2 insulin receptor-like protein is also activated. The AKT-1 and AKT-2 kinases, as

well as molecules from the parallel pathway, negatively regulate DAF-16 activity, possibly via phosphorylation. Phosphorylated DAF-16 could be inactive, function to activate genes required for reproductive growth and metabolism, or repress genes required for dauer arrest and energy storage. Other signaling molecules that are activated by DAF-2 must also converge downstream of AGE-1 (for example, on DAF-16 or AKT-1/AKT-2) for proper regulation of metabolism and lifespan: the dauer arrest induced by loss of AGE-1 PI3K or AKT-1/AKT-1 activity implies that the loss of only one of these inputs to DAF-16 is sufficient to cause dauer arrest. Under dauer inducing conditions, DAF-2, AGE-1, AKT-1/AKT-2, and other signaling pathways from DAF-2 are inactive and therefore DAF-16 is active, presumably because it is under-phosphorylated. Active DAF-16 either represses genes required for reproductive growth and metabolism or activates genes necessary for dauer arrest and energy storage.

The DAF-16 Fork head protein has been suggested to interact with the DAF-3, DAF-8, or DAF-14 Smad proteins to integrate converging TGF- $\beta$  like neuroendocrine signals with insulin-like signals (Ogg et al., *Nature* 389:994-999, 1997; Patterson et al., *Genes & Development* 11:2679-2690, 1997). DAF-16 may form a complex with the DAF-3 Smad protein under dauer inducing conditions to regulate these downstream genes (Ogg et al., *Nature* 389:994-999, 1997), while AKT-1 phosphorylation of DAF-16 may inhibit the formation of a Smad/Fork head complex during reproductive development.

Akt/PKB has been implicated in mammalian insulin receptor signaling that localizes glucose transporters to the plasma membrane (Kohn et al. (1996) *J. Biol. Chem.* 271:31372-31378) and has been shown to regulate glycogen synthesis via direct phosphorylation of GSK3 (Cross et al. (1995) *Nature* 378:785-789); two

events that are not transcriptionally regulated. While there also may be such Akt/PKB outputs in *C. elegans*, the DAF-16 Fork head transcription factor represents the major output of DAF-2/AGE-1/AKT-1/AKT-2 insulin receptor-like signaling (Ogg et al. (1997) *Nature* 389:994-999). Similarly Akt/PKB action in the insulin/IGF-I anti-apoptotic pathway (Dudek et al. (1997) *Science* 275:661-665; Kauffmann-Zeh et al. (1997) *Nature* 385:544-548; Kulik et al. (1997) *Mol. Cell Biol.* 17:1595-1606) may also converge on transcription factors related to DAF-16.

The present model, based on genetic evidence that Akt/PKB couples insulin receptor-like signaling to transcriptional output via the DAF-16 Fork head transcription factor in *C. elegans*, predicts that Akt/PKB will have transcriptional outputs in insulin-like signaling across phylogeny. It was previously suggested that the human homologs of the DAF-16 transcription factor (AFX, FKHR, FKHL1 and AF6q21) may be the pertinent downstream effectors of insulin signaling in humans (Ogg et al., *Nature* 389:994-999, 1997). Two of the consensus Akt/PKB sites conserved in DAF-16 and its human homologs are located outside of the Fork head DNA binding domain, and two sites are located in the highly basic W2 region of the Fork head domain that has been shown to mediate DNA phosphate backbone contacts (Clark et al. (1993) *Nature* 364:412-420). Insulin stimulated Akt/PKB phosphorylation of the W2 sites may affect DNA binding whereas the other conserved sites may affect transactivation. A recent report shows that Akt/PKB mediates insulin dependent repression of the insulin-like growth factor binding protein-1 (IGFBP-1) gene in HepG2 cells via a conserved insulin response sequence (CAAAAC/TAA) (Cichy et al., *J. Biol. Chem.* 273:6482-6487, 1998). Interestingly, we have determined that DAF-16

binds to this same insulin response sequence *in vitro*. We propose that Akt/PKB mediates its transcriptional effects on insulin responsive genes such as IGFBP-1 via the human homologs of DAF-16: AFX, FKHR, FKHL1, or AF6q21.

In addition, genetic analysis suggests that drugs that activate AKT or PDK can bypass the need for AGE-1 PI3K signaling, and mapping of mutations to particular regions of AKT-1 and PDK-1 points out targets for activation of these enzymes. Thus, drugs that activate these kinases are expected to partially relieve defects in insulin signaling, for example, associated with type II diabetes. The genetic analysis described herein also suggests that another unknown output of DAF-2 insulin like signaling exists. That output may be identified using AKT gain of function mutations to activate the AGE-1 PI3K pathway and screening for mutations that allow *daf-2* receptor mutations to grow reproductively. Alternatively, the genes in this parallel pathway may be identified by screening *age-1;daf-18* mutants for arrest at the dauer stage.

### **PDK genetics**

From the same genetic screen that generated the *akt-1(mg144gf)* allele, we identified another *age-1* suppressor, *mg142*. This mutation also bypasses the need for upstream *age-1* signaling and is genetically dominant. Genetic mapping placed the mutation in the region where a *C. elegans* homologue maps. The genomic sequence of *pdk-1*, starting 60 bp upstream of the start codon and ending 60 bp downstream of the stop codon is shown in Figure 35 (SEQ ID NO: 158). Figures 36 and 37 show the two *C. elegans pdk-1* spliced forms, *pdk-1a* (Figure 36; SEQ ID NO: 159) and *pdk-1b* (Figure 37; SEQ ID NO: 160). The *pdk-1(mg142)* gain of function mutation is Ala303Val (splice 1). This protein is

58% identical to mammalian PDK in the plecstrin homology domain and 39% identical in the kinase domain as shown below (SEQ ID NOS: 170-199).

Score = 252 (88.7 bits), Expect = 2.2e-60, Sum P(6) = 2.2e-60  
Identities = 47/80 (58%), Positives = 60/80 (75%), Frame = +3

5 Query: 439 LEQAGGNPWHQFVENNLILKMGPVDKRGKGLFARRRQLLLTEGPHLYYVDPVNVKVLKGEI 498  
LE+Q NP+H F N+LILK G ++K++GLFARRR LLTEGPHL Y+D N VLKGE+  
Sbjct: 1818 LEBQRVKNPFHIFTNNSLILKQGYLEKKRGLFARRRMFLLTEGPHLLYIDVPNLVLKGEV 1997

Query: 499 PWSQELRPEAKNFKTFFVHT 518  
PW+ ++ E KN TFF+HT  
10 Sbjct: 1998 PWTPCMQVELKNSGTFFIHT 2057

Score = 201 (70.8 bits), Expect = 2.2e-60, Sum P(6) = 2.2e-60  
Identities = 48/123 (39%), Positives = 72/123 (58%), Frame = +1

15 Query: 263 SDLWALGCIYQLVAGLPPFRAGNEYLIQKIIKLEYDFPEKFFPKARDLVEKLLVLVDAT 322  
+D+W LGCI++Q +AG PPFRA N+Y + ++I +L++ FPE F +A +++ K+LV  
Sbjct: 802 TDIWLGCIILFQCLAGQPPFRAVNQYHLLKRIQELDFSFPPEGFPEEASEIIAKILV-G\*H 978

Query: 323 KRLGCE----EMEGYGP-----LKAHPFFESVTWENLHQQTTPKLTAYLPAMSEDE 370  
+ L E ++ P L AH FFE+V W N+ PP L AY+PA + E  
Sbjct: 979 BTLKTEYVIFNLQVRDPSTRITSQELMAHKFFENVVDWVNIANIKPPVLHAYIPATFGEPE 1158

20 Query: 371 DCYGN 375  
Y N  
Sbjct: 1159 -YYSN 1170

Score = 180 (63.4 bits), Expect = 2.2e-60, Sum P(6) = 2.2e-60  
Identities = 31/72 (43%), Positives = 52/72 (72%), Frame = +2

25 Query: 157 FGLSYAKNGELLKYIRKIGSFDETCTRFYTAEIVSALEYLHGKGIHRDLKPENILLNED 216  
F + +NG+L + + GSFD ++F+ +EI++ L++LH I+HRD+KP+N+L+ +D  
Sbjct: 287 FVIGLVENGDLGESLCHFGSFDMLTSKFFASEILTGLQFLHDNKIVHRDMKPDNVLIQKD 466

Query: 217 MHIQITDFGTAK 228  
HI ITDFG+A+  
Sbjct: 467 GHILITDFGSAQ 502

30 Score = 83 (29.2 bits), Expect = 2.2e-60, Sum P(6) = 2.2e-60  
Identities = 15/53 (28%), Positives = 32/53 (60%), Frame = +2

Query: 108 YAIKILEKRHIKENKVPIVTRERDVMSRLD----HPFFVKLYFTFQDDEKL 155  
+A+K+L+K ++ + K+ + RE+++++ L HPF +LY F D ++  
Sbjct: 8 FAVKVLQKSYLNRHQMDAIIREKNILTYLSQECGGHPFVTQLYTHFHDQARI 166

35 Score = 81 (28.5 bits), Expect = 2.2e-60, Sum P(6) = 2.2e-60  
Identities = 15/29 (51%), Positives = 19/29 (65%), Frame = +2

Query: 519 PNRTYYLMDPSGNAHKWCRKIQEVWRQRY 547

PNR YYL D A +WC+ I +V R+RY  
 Sbjct: 2129 PNRVYYLFDLEKKADEWCKAINDV-RKRY 2212

Score = 78 (27.5 bits), Expect = 2.2e-60, Sum P(6) = 2.2e-60  
 Identities = 15/25 (60%), Positives = 18/25 (72%), Frame = +3

5 Query: 232 PESKQARANSFVGTAQYVSPPELLTE 256  
 PE AR +FVGTA YVSPE+L +  
 Sbjct: 660 PEENTARRTTFVGTAQYVSPPEMLAD 734

Overall, *C. elegans pdk-1* exhibits the following homology to human

# PDK-1.

10 Score = 118 (54.4 bits), Expect = 1.4e-104, Sum P(5) = 1.4e-104  
 Identities = 21/62 (33%), Positives = 41/62 (66%)

Query: 63 KRTSNDFMFLQSMGEGAYSQVFRCREVATDAMFAVKVLQKSYLNRHQKMDAIIREKNILT 122  
 K+ DF F + +GEG++S V RE+AT +A+K+L+K ++ + K+ + RE+++++  
 Sbjct: 76 KKRPEDFKFGKILGEGSFSTVVLARELATSREYAIKILEKRHIKENKVPYVTRERDVMS 135

15 Query: 123 YL 124  
 L  
 Sbjct: 136 RL 137

Score = 230 (106.0 bits), Expect = 1.4e-104, Sum P(5) = 1.4e-104  
 Identities = 39/90 (43%), Positives = 63/90 (70%)

20 Query: 131 HPFVTQLYTHFHDQARIYFVIGLVENGDLGESLCHFGSFDMLTSKFFASEILTGLQFLHD 190  
 HPF +LY F D ++YF + +NG+L + + GSFD ++F+ +EI++ L++LH  
 Sbjct: 139 HPFFVKLYFTFQDDEKLYFGLSYAKNGELLKYIRKIGSFDETCTRFYTAEIVSALEYLHG 198

Query: 191 NKIVHRDMKPDNVLIQKDGHILITDFGSAQ 220  
 I+HRD+KP+N+L+ +D HI ITDFG+A+

25 Sbjct: 199 KGIIHRDLKPENILLNEDMHIQITDFGTAK 228

Score = 238 (109.7 bits), Expect = 1.4e-104, Sum P(5) = 1.4e-104  
 Identities = 43/98 (43%), Positives = 67/98 (68%)

Query: 259 EENTARRTTFVGTAQYVSPPEMLADGDVGPQTDIWLGCILFQCLAGQPPFRAVNQYHLLK 318

E AR +FVGTA YVSPE+L + +D+W LGCI++Q +AG PPFRA N+Y + +  
 Sbjct: 233 ESKQARANSFVGTAQYVSPPELLTEKSACKSSDLWALGCIYQLVAGLPPFRAGNEYLIFQ 292

Query: 319 RIQELDFSFPEGFPPEEASEIIAKILVRDPSTRITSQEL 356  
 +I +L++ FPE F +A +++ K+LV D + R+ +E+

5 Sbjct: 293 KIIKLEYDFPEKFFPKARDLVEKLLVLDATKRLGCEEM 330

Score = 85 (39.2 bits), Expect = 1.4e-104, Sum P(5) = 1.4e-104  
 Identities = 17/35 (48%), Positives = 21/35 (60%)

Query: 356 LMAHKFFENVDWVNIANIKPPVLHAYIPATFGEPE 390  
 L AH FFE+V W N+ PP L AY+PA + E

10 Sbjct: 336 LKAHPFFESVTWENLHQQTTPPKLTAYLPAMSEDDE 370

Score = 324 (149.3 bits), Expect = 1.4e-104, Sum P(5) = 1.4e-104  
 Identities = 59/104 (56%), Positives = 75/104 (72%)

Query: 458 LEEQVRKNPFHIFTNNSLILKQGYLEKKRGLFARRRMFLLTEGPHLLYIDVPNLVLKGEV 517  
 LE+Q NP+H F N+LILK G ++K++GLFARRR LLTEGPHL Y+D N VLKGE+

15 Sbjct: 439 LEKQAGGNPWHQFVENNLILKMGPVDKRKGLFARRRQLLLTEGPHLYYVDPVNKVLKGEI 498

Query: 518 PWTPCMQVELKNSGTFFIHTPNRVYYLFDLEKKADEWCKAINDV 561  
 PW+ ++ E KN TFF+HTPNR YYL D A +WC+ I +V

Sbjct: 499 PWSQELRPEAKNFKTFFVHTPNRTYYLMDPSGNAHKWCRKIQEV 542

Mapping of the *mg142* mutation to this open reading frame establishes  
 20 the function of this protein. It is much more closely related to PDK than to any  
 other known kinase. PDK is a mammalian kinase that phosphorylates an essential  
 serine residue on AKT, contributing to its activation. The region of *akt-1*  
 phosphorylated by PDK-1 is shown below (SEQ ID NO: 202-207).

human AKT 276 KLENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEV 320  
 25 KLENL+LDKDGHIKI DFGLCKE I G TFCGTPEYLAPEV

Ce akt-133509 KLENLLLDKDGHIKIADFGLCKEEISFGDKTSTFCGTPEYLAPEV 33643

Ceakt2 326 LCKEEIKYGDKTSTFCGTPEYLAPEVIEDIDYDRSVDWWGVGVVYEMMCGRLPFSKENGK

LCKE I G TFCGTPEYLAPEV+ED DYT+VDWWG+GVVYEMMCGRLPF +++ +

moAKT: 298 LCKEGISDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVYEMMCGRLPFYNQDHER

5           The phosphorylated serine is conserved in *akt-1* and *akt-2*. Thus, PDK  
is an excellent candidate gene for the *mg142* mutation. The genetic region  
bearing *pdk-1* was amplified from the *mg142* strain, and an amino acid substitution  
in a conserved region of the PDK kinase domain was detected. While a gain of  
function mutation in *pdk* would be consistent with the biochemical work that  
10 shows that PDK acts upstream of AKT to activate it, this genetic work suggests  
that, if PDK can be activated (for example, by the *mg142* mutation), no PIP3  
signaling from the AGE-1 PI3K is necessary, since *mg142* suppresses an *age-1*  
null allele. To establish that this substitution causes the suppression of *age-1*  
induced dauer arrest, a strategy analogous to that used to analyze the  
15 *akt-1(mg144gf)* mutation may be utilized.

Because we have implicated PDK in the *C. elegans* insulin signaling  
pathway, human PDK1 becomes a candidate gene for variation in diabetes.  
Mutations in human PDK1 may underlie the genetic variation that causes diabetes  
in some families. Similarly, drugs that activate PDK, like the *mg142* mutation that  
20 activates *C. elegans pdk-1*, may bypass the need for upstream signaling in some  
diabetics with such upstream defects. The region of human PDK1 that is  
homologous to the *C. elegans pdk-1* at alanine 303 provides a good candidate for  
screening for drugs that bind and activate signaling. Similarly, the region of  
human AKT between the kinase domain and the PH domain, where the *C. elegans*

*akt-1* gain of function mutation maps is a good candidate for the design of drugs that activate AKT. Exemplary screens identify *daf-2* receptor mutations that are capable of reproductive growth or *age-1;daf-18* mutants that arrest at dauer stage. Such activated AKT in *C. elegans* bypasses the need for upstream signaling from the AGE-1 PI3K and may similarly treat diabetics with defects in insulin signaling between insulin and AKT.

In addition, another mutation, *pdk-1(lof)*, has been identified as a Gly to Arg substitution at position 295. This mutation causes dauer arrest in an otherwise wild-type background. This and the *mg142* mutation are located near the psuedosubstrate binding region of PDK-1, based on the crystal structure of PKA. It is likely that the G to R mutation disallows recognition of the substrate AKT-1 and AKT-2, whereas the A to V gain of function mutation may disallow recognition of a psuedosubstrate site on PDK but allow recognition of the substrate, AKT-1 and AKT-2.

Our gain of function mutations in PDK-1 and AKT-1 point to negative regulatory domains of these proteins. For example, the region flanking the *akt-1(mg144)* mutation in the nonconserved domain of *akt-1* may mediate blocking of the kinase activity, so that when this region is mutant, the kinase is more active. Similarly the region flanking the *pdk-1(mg142)* mutations in the conserved kinase domain may promiscuously activate *pdk-1*. This region is conserved in human *pdk-1* and may expose the kinase domain to the substrates, AKT-1 and AKT-2, constitutively. Chemicals that target the homologous or analogous domains in the human homologues of AKT-1, AKT-2, and PDK-1 may activate these kinases, bypassing the need for upstream insulin input and ameliorating the glucose intolerance.

### **Function of the Insulin-Like Pathway in Neurons**

In addition to the above results, we have also found that the dauer arrest and aging effects of defects in age-1 signaling can be complemented by expression of this gene in the nervous system only. We used the nervous system-specific promoter *unc-14* to drive expression of an *age-1* cDNA. The *age-1* fusion genes were placed in an *age-1* null mutant, *mg44*, which arrests at the dauer stage 100% of the time and shifts to fat storage metabolism if no maternal or zygotic *age-1* is supplied. Expression of *age-1* in just the nervous system in this mutant completely complemented the dauer arrest and long lifespan phenotypes and partially complemented the metabolic fat storage defect. The expression of *age-1* from a ubiquitous promoter, *dpy-30*, rescued all of the defects of an *age-1 mg44* null mutant. In parallel experiments, two different nervous system promoters, *unc-14* and *unc-119*, were used to drive expression of *daf-2* cDNA in *daf-2* mutant animals. However, neuronal expression of DAF-2 did not rescue the aging or metabolic phenotypes of the *daf-2* mutants. Given the multiple insulin-like ligands for DAF-2, these results may indicate that there is differential splicing of this receptor so that the cDNA introduced in these experiments supplied only one functional isoform. On the other hand, *age-1* rescues all phenotypes when expressed ubiquitously, arguing against a differential splicing mechanism.

These data indicate that the insulin signaling pathway can regulate dauer arrest from the nervous system and may also regulate aging from the nervous system. The data also show that this pathway may function as well in target tissues to regulate metabolism. It is likely that the same situation may be true of mammalian insulin like signaling: the effects of insulin on aging may be in the nervous system whereas their well known effects on muscle and adipocyte

metabolism may be akin to the DAF-2/AGE-1 regulation of metabolism from non-neuronal foci of action.

### **Diapause and Longevity**

Weak *daf-2* and *age-1* mutants that do not arrest at the dauer stage nevertheless live much longer than wild-type (Larsen et al., *Genetics* 139: 1567-1583, 1995; Kenyon et al., *Nature* 366: 461-464, 1993; Dorman et al., *Genetics* 141: 1399-1406, 1995). This connection between longevity and diapause control may not be unique to *C. elegans*. Diapause arrest is an essential feature of many vertebrate and invertebrate life cycles, especially in regions with seasonal temperature and humidity extremes (Tauber et al., *Seasonal Adaptation of Insects*, Oxford University Press, New York, N. Y., 1986). Animals in diapause arrest slow their metabolism and their rates of aging, and can survive for periods for much longer than their reproductive lifespan (Tauber et al., *supra*, 1986).

Because insulin-like DAF-2/AGE-1 signaling mediates *C. elegans* diapause longevity control, the mammalian insulin signaling pathway may also control longevity homologously. In fact, the increase in longevity associated with decreased DAF-2 signaling is analogous to mammalian longevity increases associated with caloric restriction (Finch, *Longevity, Senescence and the Genome*, The University of Chicago Press, Chicago, 1990). It is possible that caloric restriction causes a decline in insulin signaling to induce a partial diapause state, like that induced in weak *daf-2* and *age-1* mutants. The induction of diapause-like states may affect post-reproductive longevity (Finch, *supra*), as in *C. elegans*. Alternatively, it is the changes in the mode and tempo of metabolism itself rather than diapause per se that causes increased longevity. Another long-lived

*C. elegans* mutant, *clk-1*, may also regulate lifespan via such metabolic effects (Ewbank et al., *Science* 275: 980-983, 1997). This association of metabolic rate with longevity is also consistent with the correlation of free radical generation to aging (Finch, *supra*).

5     **Daf-18 Suppresses the Metabolic and Dauer Phenotypes of Age-1 and Daf-2**

          In addition to the genes described above, we have also discovered that *daf-18* functions in the insulin signaling cascade as follows. *age-1* null mutant progeny of heterozygote mothers are maternally rescued for arrest at the dauer diapause stage (Gottlieb and Ruvkun (1994) *Genetics* 137:107-120), but not for accumulation of fat (Figure 38D) or increased longevity (Gottlieb and Ruvkun  
10     (1994) *Genetics* 137:107-120). The progeny of these fat and long lived *age-1* homozygous animals, which receive no maternal or zygotic AGE-1 PI3K activity, arrest development as dauer larvae (Morris et al. (1996) *Nature* 382:536-539) (Tables VII and VIII).

15

Table VII Suppression of <i>daf-18</i> by inhibition of <i>akt-1</i> and <i>akt-2</i> gene activity					
Strain	dsRNA injected	Phenotype of Progeny at 25 °C(%)			
		L4 and Adult	Dauer	Other	N
Wild type (Bristol N2)	uninjected	99.8	0	0.2	1040
20     Wild type (Bristol N2)	<i>akt-1</i> & <i>akt-2</i>	13.7	85.9	0.3	2199
<i>daf-18(e1375)</i>	uninjected	99.1	0	0.9	1213
<i>daf-18(e1375)</i>	<i>akt-1</i> & <i>akt-2</i>	23.2	76.6	0.2	1455
<i>daf-16(mgDf50)</i>	uninjected	99.9	0	0.1	1266

5	<i>daf-16(mgDf50)</i>	<i>akt-1 &amp; akt-2</i>	97.5	0	2.5	1970
	<i>age-1(mg44)</i>	uninjected	0	99.5	0.4	228
	<i>age-1(mg44)</i>	<i>akt-1 &amp; akt-2</i>	0	94.2	5.8	277
	<i>age-1(mg44); daf-18(e1375)</i>	uninjected	99.3	0.7	0	274
	<i>age-1(mg44); daf-18(e1375)</i>	<i>akt-1 &amp; akt-2</i>	14.4	85.0	0.7	592
10	<i>daf-16(mgDf50); age-1(mg44)</i>	uninjected	100	0	0	465
	<i>daf-16(mgDf50); age-1(mg44)</i>	<i>akt-1 &amp; akt-2</i>	96.2	0	3.8	1098
	<i>daf-2(e1370)</i>	uninjected	0	99.1	0.9	109
	<i>daf-2(e1370)</i>	<i>akt-1 &amp; akt-2</i>	0	100	0	176
	<i>daf-2(e1370); daf-18(e1375)</i>	uninjected	2.2	97.8	0	225
15	<i>daf-2(e1370); daf-18(e1375)</i>	<i>akt-1 &amp; akt-2</i>	0	99.9	0.1	682
	<i>daf-16(mgDf50); daf-2(e1370)</i>	uninjected	100	0	0	487
	<i>daf-16(mgDf50); daf-2(e1370)</i>	<i>akt-1 &amp; akt-2</i>	99.1	0	0.9	780
	‘Other’ includes animals that could not be classified as dauer or non-dauer because the animal died as an embryo or young larva.					
	N, total number of animals scored.					

Table VIII Suppression of <i>age-1</i> and <i>daf-2</i> by inhibition of <i>daf-18</i> gene activity						
Strain	dsRNA injected	Phenotype of Progeny at 23°C(%)				N
		L4 and Adult	Dauer	Other		
Wild type (Bristol N2)	uninjected	100	0	0		763
Wild type (Bristol N2)	<i>daf-18</i>	99.4	0	0.6		1305
<i>age-1(m333)</i>	uninjected	0	100	0		434
<i>age-1(m333)</i>	<i>daf-18</i>	94.2	5.6	0.3		771
<i>age-1(mg109)</i>	uninjected	0	99.4	0.6		172

	<i>age-1(mg109)</i>	<i>daf-18</i>	94.7	0.3	5.0	341
	<i>age-1(mg44)</i>	uninjected	0	97.9	2.1	389
	<i>age-1(mg44)</i>	<i>daf-18</i>	90.7	7.1	2.1	701
	<i>age-1(mg44); daf-18(e1375)</i>	uninjected	99.8	0	0.2	569
5	<i>daf-2(e1370)</i>	uninjected	0	100	0	606
	<i>daf-2(e1370)</i>	<i>daf-18</i>	57.7	39.8	2.5	1266
	<i>daf-2(e1370); daf-18(e1375)</i>	uninjected	6.3	93.7	0	317
10	'Other' includes animals that could not be classified as dauer or non-dauer because the animal died as an embryo or young larva. N, total number of animals scored.					

Dauer larvae accumulate large amounts of fat (Figure 38E) and live much longer than reproductively growing animals (Klass and Hirsh (1976) Nature 260:523-525). The dauer arrest (Gottlieb and Ruvkun (1994) Genetics 137:107-120; Larsen et al. (1995) Genetics 139:1567-1583; Tables VII and VIII), fat accumulation (Figure 38F) and longevity phenotypes (Larsen et al. (1995) Genetics 139:1567-1583) of *age-1* null mutations are suppressed by *daf-18(e1375)*. *daf-18(e1375)* gene activity does not appear to interfere with normal *age-1* signaling and growth because *daf-18(e1375)* mutant animals in a wild type *age-1* background accumulate wild type amounts of fat (Figure 38B).

Although *daf-18(e1375)* behaves as a semi-dominant suppressor of *age-1*, it phenocopies inactivation of *daf-18(+)* gene activity by RNA interference (RNAi) (see below). This suggests that *daf-18(e1375)* is a loss-of-function allele that is either haploinsufficient or dominantly antimorphic. The bypass of the normal requirement for AGE-1 PI3K signaling by *daf-18(e1375)* suggests that

either lack of AGE-1 activity causes increased *daf-18* activity or that decreased *daf-18* activity increases PIP3 signals in an AGE-1-independent manner.

Although *daf-18(e1375)* readily suppresses *age-1* mutations for the metabolic, dauer, and longevity phenotypes, *daf-18(e1375)* is a less effective suppressor of *daf-2* insulin receptor-like mutations (Dorman et al. (1995) Genetics 141:1399-1406; Larsen et al. (1995) Genetics 139:1567-1583 and Tables VII and VIII). This is similar to the gain-of-function *akt-1(mg144)* which can suppress *age-1* null mutants, but not *daf-2* mutants (Paradis and Ruvkun (1998) Genes Dev. 12:2488-2498). Like the increase in *akt-1* gene activity induced by *akt-1(mg144)*, the decrease in *daf-18* gene activity caused by *daf-18(e1375)* can bypass the normal requirement for AGE-1 PI3K signaling, but not for DAF-2 insulin receptor-like signaling (Tables VII and VIII). As in the case of biochemically studied receptor tyrosine kinases, the DAF-2 receptor may have multiple parallel outputs, with AGE-1, AKT-1/AKT-2, and DAF-18 acting in one of these pathways. Signals from DAF-2 converge at the DAF-16 Fork head transcription factor, because null mutations in *daf-16* suppress all known phenotypes of *daf-2* and *age-1* null mutations (Ogg et al. (1997) Nature 389:994-999; Tables VII and VIII).

### **Daf-18 Functions Upstream of Akt-1 and Akt-2**

In contrast to the action of DAF-2 and AGE-1, AKT-1 and AKT-2 act downstream of DAF-18. *akt-1* and *akt-2* function redundantly in the regulation of dauer arrest. Inhibition of both gene activities in wild type animals by RNAi causes constitutive dauer arrest, whereas inhibition of either *akt-1* or *akt-2* alone does not (Paradis and Ruvkun (1998) Genes Dev. 12:2488-2498). Inhibition of

354041"35950350  
5 *akt-1* and *akt-2* by RNAi causes dauer arrest in either *daf-18* or wild type animals (77% and 86%, respectively, Table VII). In contrast, 0% of *daf-16(mgDf50)* animals arrest as dauers when *akt-1* and *akt-2* are inhibited (Paradis and Ruvkun (1998) Genes Dev. 12:2488-2498 and Table VII). Thus mutations in *daf-18* do not bypass the normal requirement for *akt-1* and *akt-2* activity. These data suggest that *daf-18* functions upstream or parallel to *akt-1* and *akt-2*.

10 The suppression of *age-1* null mutations by *daf-18(e1375)* is also dependent upon *akt-1* and *akt-2*. No progeny of *age-1(mg44)* null mutant homozygous animals develop to become fertile adults (99% dauer larvae, Table VII). In contrast, few *age-1(mg44); daf-18(e1375)* animals arrest as dauers (0.7% dauer larvae, Table VII), instead of developing into reproductive adults. However, when *akt-1* and *akt-2* are inhibited by RNAi in *age-1(mg44); daf-18(e1375)*, most progeny arrest at the dauer stage (85% dauers, Table VII). The weaker suppression of *daf-2* by *daf-18* is also dependent upon *akt-1* and *akt-2* (Table VII).  
15 These data suggest that the ability of *daf-18* to suppress defects in insulin-like signaling is dependent upon *akt-1* and/or *akt-2*, showing that *daf-18* acts downstream of AGE-1 PI3K, but upstream of AKT-1 and AKT-2 in this signaling cascade.

#### **Daf-18 Encodes a Homologue of Mammalian PTEN (MMAC1/TEP1)**

20 *Daf-18* maps to a genetic region (Larsen et al. (1995) Genetics 139:1567-1583) which bears the probable *C. elegans* homologue (T07A9.6) of the tumor suppressor gene PTEN (Li and Sun (1997) Cancer Res. 57:2124-2129; Li et al. (1997) Science 275:1943-1947; and Steck et al. (1997) Nat. Genet. 15:356-362). Consistent with the role of PTEN as a *daf-18* homologue is the fact that

PTEN has lipid phosphatase activity that dephosphorylates position 3 on the inositol ring of PIP<sub>3</sub> *in vitro* and decreases the levels of the lipid products of PI3K in response to insulin signaling in human 293 cells (Maehama and Dixon (1998) J. Biol. Chem. 273:13375-13378). Accordingly, a decrease in PTEN activity would  
5 be predicted to enhance PI3K signaling, consistent with *daf-18* activity. Genetic mapping, the detection of the *daf-18(e1375)* mutation in this PTEN homologue, and the similar phenotype to *daf-18(e1375)* caused by RNAi of this PTEN homologue all demonstrate that *daf-18* corresponds to this gene.

The sequence of a full length *daf-18* cDNA predicts a protein of 962  
10 amino acids (Figures 40A and 40B). Homology between DAF-18 and human PTEN (U93051; Li et al. (1997) Science 275:1943-1947) is highest within the phosphatase domain (38% identical, 94/250 aa) which is located at the amino-terminal end of both proteins (Figures 39A and 39B). Amino acids surrounding the probable active site Cys-(X)<sub>5</sub>-Arg sequence are 90% identical  
15 (18/20 aa) between DAF-18 and PTEN (Figure 39B). This suggests that the substrate specificity of DAF-18 and PTEN may be similar.

Using the canonical *daf-18* PTEN cDNA sequences and genomic sequence from the *C. elegans* Genome Sequencing Consortium, the coding region and intron/exon boundaries of *daf-18* were sequenced in *daf-18(e1375)* and  
20 compared to the sequence of wild type. A 30 base pair insertion mutation was detected in *daf-18(e1375)* (Figure 39A). This insertion mutation occurs within exon 4 and is predicted to insert 6 amino acids to the coding sequence before introducing a stop codon. The insertion is composed of a thirteen base pair repeat and two smaller repeat segments. The mutation is predicted to leave the  
25 phosphatase domain intact, but to truncate the carboxy-terminal half of the protein.

Since the mutation maps to an unconserved domain and because inhibition of *daf-18* by RNAi is more severe than *daf-18(e1375)* (see below), it is unlikely that *daf-18(e1375)* is a null mutant.

Although many of the oncogenic human PTEN mutations map to the phosphatase domain, several have been identified in the carboxy-terminal half of the protein (see the Human Gene Mutation Database and references therein; Krawczak and Cooper (1997) Trends Genet. 13:121-2). These carboxy-terminal mutations are analogous to *daf-18(e1375)*. Since some oncogenic mutations in PTEN and the *daf-18(e1375)* allele are localized to the carboxyl-terminal end, these regions, though unconserved between *C. elegans* and mammals, may be critical for phosphatase localization or function.

*Daf-18(e1375)* is the only identified *daf-18* allele, despite the extensive genetic screens that have been done for genes in the *daf* pathway. Additional *daf-18* alleles have not been isolated in screens for suppressors of *daf-2* (in contrast to the scores of *daf-16* alleles), which may be due to the weak suppression of *daf-2* by *daf-18(e1375)* (Tables VII and VIII). Because of the strong suppression of *age-1* null mutants by *daf-18(e1375)*, more alleles would be expected from screens for *age-1* suppressor mutations.

The *daf-18(e1375)* allele causes other phenotypes besides suppression of the *age-1* null mutant. 8% of *daf-18(e1375)* animals (n=831) die as adults with a burst vulva compared to 0% of wild type (Bristol N2) adults (n=920) grown at 23°C. This suggests that *daf-18* may function in other signal transduction pathways. Consistent with this, a *daf-18* promoter::green fluorescent protein fusion is expressed in many tissues throughout the animal.

### Inactivation of Daf-18 by RNAi Suppresses Age-1 and Daf-2

Inactivation of the *C. elegans* PTEN homologue T07A9.6 by RNAi confirms the assignment of *daf-18* to the gene and the assignment of *daf-18* to a function downstream of *age-1* PI3K and upstream of *akt-1* and *akt-2*. The  
5 inactivation of *daf-18* by RNAi potently suppresses null mutations in *age-1* and more weakly suppresses a *daf-2* insulin receptor-like mutant. Whereas the homozygous progeny of three different *age-1* mutant alleles, including two null alleles, arrest at the dauer stage virtually 100% of the time (Dorman et al. (1995) Genetics 141:1399-1406; Larsen et al. (1995) Genetics 139:1567-1583 and Table  
10 VIII), inhibition of *daf-18* by RNAi suppresses the dauer constitutive phenotype of *age-1(m333)*, *age-1(mg109)* and *age-1(mg44)* (only 6%, 1% and 8% dauers, respectively) (Table VIII). This is comparable to the suppression of *age-1(mg44)* by *daf-18(e1375)* (0% dauers, Table VIII).

Inhibition of *daf-18* PTEN by RNAi partially suppresses a  
15 loss-of-function allele of the *daf-2* insulin receptor-like gene. This suppression is most easily observed under conditions where *daf-2* gene activity is decreased, but probably not missing. *daf-2(e1370)* is a temperature sensitive allele with a mutation in the kinase domain (Kimura et al. (1997) Science 277:942-946). At a low temperature (15°C), *daf-2(e1370)* animals do not form dauers, but more  
20 restrictive temperatures (25°C or 23°C) cause 100% arrest at the dauer stage (Tables VII and VIII). The arrest of *daf-2(e1370)* at 23°C is weakly suppressed by *daf-18(e1375)* (94% dauers), but inhibition of *daf-18(+)* by RNAi suppresses *daf-2(e1370)* much more potently (40% dauers) (Table VIII). At 25°C, *daf-18(e1375)* is a weaker *daf-2* suppressor, suggesting that DAF-2 insulin  
25 receptor-like outputs, parallel to the AGE-1 PI3K, DAF-18 PTEN, and AKT-1/2

pathways, are more essential at this higher temperature. In contrast, the *daf-16(mgDf50)* null mutation completely suppresses *daf-2(e1370)* at all temperatures (0% dauers, Tables VII and VIII). This suggests that divergent signals from DAF-2 (AGE-1/DAF-18/AKT-1/2 and another putative pathway) converge upon DAF-16.

These results suggest that *daf-18(e1375)* is a partial loss-of-function mutation and that inhibition of *daf-18* by RNAi causes a larger decrease in *daf-18* gene activity. Similar to *daf-18(e1375)*, the inhibition of *daf-18* gene activity in wild type causes some animals to burst at the vulva, but no other obvious phenotypes. The inhibition of *daf-18* gene activity by RNAi, however, does not necessarily reveal the phenotype induced by the complete loss of *daf-18* gene activity.

#### **Assignment of Daf-18 to the DAF-2 Signaling Pathway**

Our assignment of the *daf-18* molecular function to a homologue of the PTEN lipid phosphatase fits into our genetic analysis of its action in the DAF-2 insulin receptor-like signaling pathway. The genetic pathway analysis shows that DAF-18 is likely to act between the AGE-1 PI3K and AKT-1/AKT-2. Because PTEN has been shown to dephosphorylate position 3 of the inositol ring of PIP3, DAF-18 may modulate DAF-2 signals by decreasing the PIP3 output of AGE-1 PI3K. DAF-18 may normally decrease the level of PIP3 signals, perhaps insulating signals emanating from the DAF-2/AGE-1 signaling complex from other PIP3 signals in the cell, or resolving insulin-like signaling episodes by restoring lipid levels to pre-insulin status. Perhaps the long carboxyl-terminal tail region of DAF-18 PTEN mediates its localization to insulin signaling complexes,

insulating them from other signaling complexes, or vice versa. Loss of DAF-18 would be expected to enhance PIP3 signaling to the Akt kinases by allowing the second messenger to promiscuously signal between receptor complexes.

It is not clear from the genetic analysis whether DAF-18/PTEN activity is regulated during insulin-like or other signaling. For example, there may be phosphorylation input to activate or inactivate DAF-18 activity. One attractive possibility is that DAF-18 becomes activated by Akt or PDK1 as a component in the recovery from an episode of insulin signaling. It may be significant that PTEN lipid phosphatase activity in vitro is low (Maehama and Dixon (1998) J. Biol. Chem. 273:13375-13378), perhaps due to a missing modification by the insulin signaling cascade.

DAF-18 may also be regulated by a TGF- $\beta$  signaling pathway. In *C. elegans* a TGF- $\beta$  signaling pathway converges with the DAF-2 insulin receptor-like signaling pathway (Ogg et al. (1997) Nature 389:994-999) and PTEN expression has been reported to be downregulated by TGF- $\beta$  signaling in cell culture (Li and Sun (1997) Cancer Res. 57:2124-2129). The *C. elegans* DAF-7 TGF- $\beta$  and insulin-like signaling pathways are also synergistic, whereby declines in the TGF- $\beta$  signals enhance the mutant phenotypes caused by declines in insulin-like signals (Ogg et al. (1997) Nature 389:994-999). If DAF-18 PTEN expression is similarly responsive to DAF-7 TGF- $\beta$  inputs, its activity may mediate cross talk between these pathways in metabolic control.

The molecular assignment of DAF-18 to the PTEN lipid phosphatase rationalizes *daf-18* genetic activities in *C. elegans* metabolic control and longevity. Reduction of *daf-18* gene activity causes a decrease in fat storage in an *age-1* mutant, perhaps because the ensuing activation of AKT-1 and AKT-2 mimics that

induced by insulin-like signaling, causing a shift from fat storage metabolism to reproductive, perhaps sugar-based, metabolism. The *daf-18(e1375)* mutation also strongly suppresses the longevity increase caused by the weak *age-1(hx546)* PI3K mutation or the weak *daf-2(e1370)* insulin receptor-like mutation at a semi-permissive temperature (Dorman et al., (1995) Genetics 141:1399-1406; Larsen et al. (1995) Genetics 139:1567-1583), whereas *daf-18(e1375)* only weakly suppresses the longevity increase caused by null *age-1* mutations or *daf-2(e1370)* at the non-permissive temperature (Dorman et al., (1995) Genetics 141:1399-1406; Larsen et al. (1995) Genetics 139:1567-1583). These data show that even though the increase in PIP<sub>3</sub> levels caused by a decrease in *daf-18* gene activity can bypass the need for AGE-1 signaling in dauer arrest, the resulting level of PIP<sub>3</sub> is not sufficient to induce normal aging. These results are congruent with aging and dauer arrest phenotypes of an *age-1* allelic series: the highest levels of *age-1* (i.e., PIP<sub>3</sub>) are necessary for normal longevity, whereas animals with decreased but non-zero levels of PIP<sub>3</sub> age more slowly, but do not arrest at the dauer stage. And only when both zygotic and maternal AGE-1 is missing do PIP<sub>3</sub> levels decline to the point that animals arrest at the dauer stage (Gottlieb and Ruvkun (1994) Genetics 137:107-120; and Riddle (1988) The Dauer Larva. In The Nematode *Caenorhabditis elegans*, W.B. Wood, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), pp. 393-412). We have not yet determined whether the regulation of metabolism is the cause of the longevity phenotype (or vice versa) or represents a co-regulated output of the DAF-2 insulin receptor-like pathway.

This genetic behavior is similar to that of activated AKT-1, which can suppress the dauer arrest caused by complete lack of AGE-1 PI3K signaling, but not the longevity increase (Paradis and Ruvkun (1998) Genes Dev. 12:2488-2498).

The suppression of the *age-1* null mutant metabolic phenotypes by *daf-18*, but not by the *akt-1* gain-of-function mutation, suggests that an increase in PI(3,4)P<sub>2</sub> and PIP<sub>3</sub> levels is a closer mimic to wild type than activated AKT-1, perhaps because both AKT-1 and AKT-2 are activated by increased lipid signaling in a *daf-18* mutant. It may also be significant that declines in *daf-18* activity and the presumed concomitant increase in PI(3,4)P<sub>2</sub> and PIP<sub>3</sub> levels in wild type has a negligible effect on longevity (Dorman et al., (1995) Genetics 141:1399-1406; Larsen et al. (1995) Genetics 139:1567-1583). Presumably, once PIP<sub>3</sub> levels are above a threshold, increasing their levels does not influence lifespan.

Our molecular model suggests that DAF-2, AGE-1, DAF-18, AKT-1, AKT-2, and DAF-16 act in the same cells. It has not addressed whether these genes in fact act in the same cells nor have we discerned whether this pathway acts in key endocrine signaling cells or in target tissues. *daf-18*, *akt-1*, and *daf-16* are all expressed in neurons and throughout much of the animal (Ogg et al. (1997) Nature 389:994-999; Paradis and Ruvkun (1998) Genes Dev. 12:2488-2498), consistent with their function either in signaling cells or target tissues.

Inhibition of *daf-18* can suppress *age-1* mutations (*m333* and *mg44*) that are predicted to truncate AGE-1 before the kinase domain and therefore generate no PIP<sub>3</sub> at all (Morris et al. (1996) Nature 382:536-539; Table VIII). The ability of *daf-18* inhibition to suppress *age-1* null mutations, and our demonstration that *daf-18* suppression depends on the Akt kinases, suggests that there must be another source of PI(3,4)P<sub>2</sub> or PIP<sub>3</sub>. This alternative source of lipids is not normally redundant with those generated by DAF-2/AGE-1 signaling because *age-1* mutations have metabolism, reproductive growth, and lifespan phenotypes. In the absence of *daf-18*, lipids may accumulate to levels sufficient to activate the Akt

kinases.

In addition to AGE-1, there are two other PI3K genes in the *C. elegans* genome. AGE-1 is the only member of the "Type I" class that includes the 110-130 kilodalton catalytic/50-85 or 101 kilodalton adaptor heterodimers (Vanhaesebroeck et al. (1997) TIBS 22:267-272). Members of this class of PI3Ks are activated by growth factors and heterotrimeric GTP-binding protein-coupled receptors and phosphorylate phosphatidylinositol, PI(4)P, and PI(4,5)P<sub>2</sub> to generate PI(3)P, PIP(3,4)P<sub>2</sub> and PIP<sub>3</sub> in vitro. PIP<sub>3</sub> may be dephosphorylated at the 5-position to yield the actual PI(3,4)P<sub>2</sub> signal (Damen et al. (1996) Proc. Natl. Acad. Sci. U.S.A. 93:1689-93; Ono et al. (1996) Nature 383:263-6). The "type II" class is represented in *C. elegans* by F39B1.1. Members of this class are defined by amino terminal extensions and a C2 domain at their carboxy termini (Newton (1995) Curr. Biol. 5:973-6; Vanhaesebroeck et al. (1997) TIBS 22:267-272). C2 domains were originally described as Ca<sup>2+</sup>-dependent phospholipid binding motifs, but they have been found to bind lipid in a Ca<sup>2+</sup>-independent manner and may also mediate protein-protein interactions. Type II PI3Ks preferentially phosphorylate phosphatidylinositol and PI(4)P, over PI(4,5)P<sub>2</sub>, to generate PI(3)P and PI(3,4)P<sub>2</sub>. "Type III" PI3Ks are related to yeast protein VPS34 (Vanhaesebroeck et al. (1997) TIBS 22:267-272). These proteins have regulatory subunits and phosphorylate phosphatidylinositol, exclusively. Rather than being activated by cellular agonists, type III PI3Ks are thought to participate in vesicle sorting (De Camilli et al. (1996) Science 271:1533-9). The *C. elegans* gene, B0025.1, is most closely related to this family.

Since AGE-1 is the only known *C. elegans* type I PI3K, the type II PI3K, F39B1.1, may be the alternative source of 3-phosphorylated

phosphoinositides which can activate Akt. DAF-18 may normally insulate DAF-2/AGE-1 signaling from other PI(3,4)P<sub>2</sub> or PIP<sub>3</sub> second messenger signals in the cell. But when DAF-18 activity is inhibited, cross talk from this other PI3K may promiscuously activate the Akt kinases which are normally dependent on AGE-1 PI3K generated PIP<sub>3</sub>.

Our placement of DAF-18/PTEN downstream from AGE-1 PI3K and upstream from AKT-1 and AKT-2 suggests that mammalian PTEN may also regulate Akt activity by modulating PI3K signals. In fact, recent experiments with PTEN knockout mutant mice have shown that PTEN acts upstream of Akt in mammalian growth factor signaling pathways (Stambolic et al. (1998) Cell 95:29-39). More specifically, the action of DAF-18 PTEN in the *C. elegans* insulin signaling metabolic and longevity control pathway suggests that mammalian PTEN may modulate insulin control of metabolism and lifespan. Reduction in PTEN activity would be expected to potentiate insulin and/or insulin-like growth factor signaling, but an increase of PTEN activity would be expected to cause insulin resistance downstream of the insulin receptor, the type observed in late onset diabetes. Thus PTEN on chromosome 10 is a candidate gene for human autosomal dominant type II diabetes as well as for human longevity control.

### Methods

The experiments described above were carried out using the following materials and methods.

### Strains

Alleles used were as follows: LG I *daf-16(mgDf50)*; LG II *sqt-1(sc13)*

*age-1(mg44)/mnC1, unc-4(e120) age-1(m333)/mnC1, unc-4(e120)*  
*age-1(mg109)/mnC1; LGIII daf-2(e1370).*

### **Sudan Black Staining**

Larvae and young adults maintained in well fed conditions were washed  
5 in M9 buffer (Brenner (1974) Genetics 77:71-94) for 30 minutes, fixed in M9 with  
1% paraformaldehyde, and subjected to three freeze thaws. Animals were then  
washed and dehydrated through washes with 25%, 50% and 70% ethanol.  
Staining was performed overnight in a 50% saturated solution of Sudan Black B in  
70% EtOH. Stained animals were visualized with a Zeiss Axioplan microscope.

### **RNA Interference (RNAi) and Dauer Arrest Assays**

*akt-1* and *akt-2* RNAi was performed as described (Paradis and Ruvkun  
(1998) Genes Dev. 12:2488-2498). *daf-18* RNAi was performed in a similar  
manner. The full length *daf-18* cDNA was amplified by PCR from yk400b8 (Y.  
Kohara) using primers CM024 and CM025 (Paradis and Ruvkun (1998) Genes  
15 Dev. 12:2488-2498). RNA was transcribed using MEGAscript T3 and T7 kit  
(Ambion) and then single stranded RNA was combined prior to injection. L4  
hermaphrodites or young adults were injected into the gut with approximately 5  
 $\mu\text{g}/\mu\text{l}$  double stranded RNA and then were allowed to recover overnight at the  
experimental temperature. To assay dauer arrest, single injected animals or  
20 uninjected L4 hermaphrodites or young adults were moved to new plates and again  
on the next two subsequent days. All progeny laid after the recovery period were  
scored two days after being laid as dead eggs, dauer larvae, L4 larvae, adults or  
animals with aberrant development. "Dauers" included dauers and partial dauers

as defined (Paradis and Ruvkun (1998) Genes Dev. 12:2488-2498). For the experiments with *age-1* mutants, *age-1* homozygous mutant progeny of *age-1* heterozygous mothers were injected.

### **Sequencing**

5 Genomic DNAs from *daf-18(e1375)* and wild type (Bristol N2) were PCR amplified and directly sequenced. A putative full length clone, yk400b8 (gift from Y. Kohara), was fully sequenced. The sequence of this clone and additional clones partially sequenced by Y. Kohara (yk423e3, yk400b8, yk419d6, yk282b4, yk226d6, yk219b10, yk200a11, yk181h9, yk49a4, and yk43e5) have a different  
10 exon/intron structure than was predicted for T07A9.6 by Genefinder.

### **Drugs that Regulate DAF-18 PTEN Lipid Phosphatase in the Treatment of Diabetes, Obesity, and Aging**

Since DAF-18/PTEN is a lipid phosphatase, chemical modulation of its activity may be readily identified using any standard *in vitro* lipid phosphatase  
15 assay (see, for example, Maehama and Dixon, *J. Biol. Chem.* 273:13375, 1998). Chemicals identified by this initial screen may then be tested in a *C. elegans* assay as described herein. These tests are best done using the human homologue of DAF-18, the oncogene PTEN, both in vitro and transformed into a *C. elegans* strain lacking *daf-18* gene activity (also as described herein). In particular,  
20 chemicals that activate human PTEN in vitro may be tested on *C. elegans daf-18* mutants expressing human PTEN from the *daf-18* promoter, assaying for dauer arrest, metabolic switch from fat storage, and/or increased longevity, either in an otherwise wild type background or in an *age-1* or *daf-2* mutant background. If

desired, chemicals that perturb longevity or metabolism of such humanized *C. elegans* could also be tested on mice.

These chemicals would be expected to affect glucose and fat levels and treat type II diabetes and obesity. In particular, chemicals that activate DAF-18 would be expected to increase longevity. In addition, even though such chemicals could affect the cell cycle, since PTEN is a recessive oncogene, skin creams that activate PTEN would be expected to have youth enhancing activities. Conversely, chemicals that inhibit DAF-18 activity would be expected to treat type II diabetes and obesity, consistent with the fact that decreases in DAF-18 gene activity completely bypass the need for *age-1* PI3 kinase signaling and partially bypass the need for *daf-2* insulin receptor-like signaling. Thus, drugs that inhibit human PTEN activity *in vitro* are preferably tested on *C. elegans* for the ability to bypass the need for *age-1* PI3K signaling in an animal carrying human PTEN expressed from the *daf-18* promoter. In one particular example, any drug that inhibited human PTEN activity would allow an *age-1(0); daf-18(0)* mutant strain carrying human PTEN expressed from the *daf-18* promoter to grow reproductively, rather than arresting in a manner characteristic of the parent strain. Thus, drugs shown to inhibit human PTEN *in vitro* could be tested on worms of the *age1(0); daf-18(0); daf-18 promoter/PTEN* genotype for ability to allow reproductive growth. If desired, such drugs could then be tested for diabetic therapeutic efficacy in mouse or rat models of obesity onset diabetes (as described herein). Drugs identified by this screen would treat some type II diabetic patients as well as some obese patients with defects in the PI3K outputs of the insulin receptor pathway.

**Glucose Regulation by the *C. elegans* Insulin Like Signaling Pathway:**

### Confirmation of its Applicability to Human Diabetes

We have constructed a full length protein fusion of GFP to a highly expressed glucose transporter orthologue in the worm genome: H17B01. The H17B01.1 (GLUT) GFP fusion was amplified with primer CAW59

5 (ccactatggccgagatttc) and CAW60 (ccagtgaaaagttctctcttctctctctctcgaattcgga). CAW59 is the promoter primer and corresponds to nucleotides 31101-31120 in cosmid H17B01 and 39249-39268 in YAC Y51H7.contig253. Primer CAW60 is the GFP-fusion primer. The first 23 nucleotides are GFP and the last 21 are GLUT bottom strand (i.e.cttctctctctcgaattcggc) corresponding to 48128-48108 in  
10 Y51H7.contig253 and 5015-5035 in C13F7 (the cosmid that joins H17B01). The protein sequence is as follows (SEQ ID NO: 208):

MGVNDHDVSVPLQEVQSRTVEGKLTCLAFSAFVITLASFQFGYHIGCVN  
APGGLITEWIIGSHKDLFDKELSRENADLAWSVAVSVFAVGGMIGGLSSG  
WLADKVGRRGALFYNNLLALAAAALMGLAKSVGAYPMVILGRLIIGLNCG  
15 FSSALVPMFLTEISPNNLRGMLGSLHQLLVITAILVSQIFGLPHLLGTGD  
RWPLIFAFTVVPVAVLQLALLMLCPESPKYTMVARGQRNEAESALKKLRDT  
EDVSTEIEAMQEEATAAGVQEKPKMGDMFKGALLWPMSIAIMMMLAQQLS  
GINVAMFYSTVIFRGAGLTGNEPFYATIGMGAVNVIMTLISVWLVDHPKF  
GRRSLLLAGLTGMFVSTLLLVGALTIQNSGGDKWASYSAGFVLLFVISF  
20 ATGPGAIPWFFVSEIFDSSARGNANSIAVMVNWAANLLVGLTFLPINNLM  
QQYSFFIFSGFLAFFIFTWKFPETKGSIEQIQAEFEKRRK

The predicted coiled-coil domain is from 237-258 (SEQ ID NO: 209):

RNEAESALKKLRDTEDVSTEIE

This transporter contains a coiled coil domain in common with the glut4 insulin

responsive mammalian glucose transporter and the glut1 mammalian thrombin responsive glucose transporter of platelets. This coiled coil domain may mediate the tethering of these subfamily of glucose transporters adjacent to the plasma membrane so that these transporters can be fused upon triggering signals, for example, from insulin.

We have verified that the localization of the H17B01 glucose transporter is responsive to *daf-2* insulin like signaling. In particular, the transporter is suspended in vesicles in a *daf-2* mutant but is placed in the cell membrane in wild type animals with normal insulin like signaling. The insulin responsive fusion of these transporters with the cell membrane is most easily observed in the nervous system of *C. elegans*. This discovery endorses the glucoregulatory role of DAF-2 insulin like signaling in *C. elegans*, further confirming the orthology with mammalian insulin regulation of glucose transport. It also points out a possible regulatory role for glucose transport in the nervous system. It is possible that the regulation of sugar metabolism by insulin in the brain may be more important in humans than has previously been appreciated. The study of human insulin responses have been focused on peripheral tissues, but it is entirely possible that the central responses to insulin are key in the disease progression.

We have also shown that the glucose transporter genes of *C. elegans* are transcriptionally responsive to insulin signaling. The promoter of this gene is a good candidate for finding DAF-16 binding sites and DAF-3 binding sites. In mammals, glucose transporters are transcriptionally regulated by insulin signaling, suggesting that the connection between DAF-16 and the glucose transporter may be general to the DAF-16 homologues, AFX, FKHR, and FKHL1 and mammalian glucose transporters such as Glut4 whose transcription is regulated by

insulin. Indeed we find that the expression of the glucose transporter GFP fusion is downregulated in starved wild type animals but is not so downregulated in *daf-16* mutant animals, suggesting that it is *daf-16* activity that represses the expression of this gene.

5     **Synergistic Control of Metabolism and Diapause by Insulin and TGF-β Signaling Pathways**

          In addition to DAF-2 signaling, the DAF-7 TGF-β neuroendocrine signal is also necessary for reproductive development of *C. elegans* (Ren et al., *Science* 274: 1389-1391, 1996; Schackwitz et al., *Neuron* 17: 719-728, 1996).

10    The signals in these two pathways are not redundant: animals missing either *daf-2* signaling or *daf-7* signaling (Fig. 3) shift their metabolism and arrest at the dauer stage (Table IX). In addition the phenotypes caused by mutations in either pathway are strongly synergistic, suggesting that the two pathways are integrated. Synchronised eggs were grown and counted as described above. *daf-1(m40)* and  
15    *daf-2(e1370)* form 100% dauer at 25°C. Numbers shown in Table IX indicate percentage dauer formation and number of animals counted (in parenthesis). Data presented is the sum of three independent trials.

Table IX. Synergy of *daf-1* and *daf-2*

	% dauer formation	
	15°C	20°C
<i>daf-1</i> (m40)	0.0 (532)	1.9 (909)
<i>daf-2</i> (e1370)	0.0 (798)	3.8 (503)

% dauer formation

<i>daf-1</i> (m40); <i>daf-2</i> (e1370)	19.4 (747)	100 (718)
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This data indicates that DAF-7 TGF- $\beta$  signals and DAF-2 ligand insulin-like signals are integrated. In support of this model, weak mutations in the *daf-2* insulin signaling pathway and in the *daf-7* TGF- $\beta$  signaling pathway are highly synergistic (Table IX). Genetic epistasis analysis indicates that the DAF-7 and DAF-2 pathways are parallel rather than sequential (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994). That is, *daf-16* mutations strongly suppress *daf-2* mutations but not *daf-7*, *daf-1*, or *daf-4* mutations, whereas *daf-3* mutations strongly suppress *daf-7*, *daf-1*, and *daf-4* mutations, but not *daf-2* mutations. Analogous synergism between activin and FGF tyrosine kinase pathways in *Xenopus* mesoderm induction has been noted (Green et al., *Cell* 71: 731-739, 1992).

A dauer-inducing pheromone regulates the production of DAF-7 by the ASI sensory neuron (Ren et al., *Science* 274: 1389-1391, 1996; Schackwitz et al., *Neuron* 17: 719-728, 1996). Because animals carrying *daf-7* nonsense or truncation mutations are responsive to pheromone (Golden and Riddle, *Proc. Natl. Acad. Sci. U.S.A.* 81: 819-823, 1984), we further suggest that the production of the insulin-like ligand for DAF-2 is also regulated by pheromone. It is not yet clear whether these DAF-7 and DAF-2 signals converge in target tissues or in other regulatory (i.e., hormonal) cells; however the expression of the DAF-7 receptor pathway genes in essentially all target tissues (infra) suggests that integration

occurs there.

### **DAF-7 and Diabetes**

Based on the data herein, we propose that in humans as in *C. elegans*, both a DAF-7-like neuroendocrine signal and insulin are necessary for metabolic control by insulin. According to this model, the failure of target tissues to respond to insulin signals in Type II diabetic patients could be due to defects either in the insulin or TGF- $\beta$ -like control pathways. Pedigree analysis has shown a strong genetic component in Type II diabetes (Kahn et al., *Annu. Rev. Med.* 47: 509-531,1996). In addition, obesity is also a major risk factor in Type II diabetes (Kahn et al., *Annu. Rev. Med.* 47: 509-531,1996). Genetic or obesity-induced (Hotamisligil et al., *Science* 259: 87-91, 1993; Lonnqvist et al., *Nat Med* 1: 950-953, 1995) declines in a DAF-7-like signaling pathway could underlie the lack of response to insulin in Type II diabetes, just as in *C. elegans daf-7* mutants cause metabolic defects very similar to *daf-2* mutants. The discovery that the DAF-7 and DAF-2 pathways converge indicates that DAF-7 hormonal signals are defective in diabetic conditions (for example, Type II diabetes), and that administration of human DAF-7 is useful for ameliorating the glucose intolerance, ketoacidosis, and atherosclerosis associated with diabetes. This is shown schematically in Figs. 17, 18, and 23.

Whereas the DAF-7 TGF- $\beta$  like and DAF-2 insulin-like signaling pathways converge to control diapause and metabolism, only the DAF-2/AGE-1 pathway has been implicated in reproductive adult stage longevity control in the absense of dauer formation (Larsen et al., *Genetics* 139: 1567-1583, 1995; Kenyon et al., *Nature* 366: 461-464, 1993; Dorman et al., *Genetics* 141: 1399-1406, 1995;

and Morris et al., *Nature* 382: 536-539, 1996). Both pathways control the longevity increase associated with dauer arrest, since dauer larvae live much longer than reproductive *C. elegans* (Riddle, In: *Caenorhabditis elegans* II, D. Riddle, T. Blumenthal, B. Meyer, J. Priess, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1997, pp. 739-768; Kenyon, op cit. pp., 791-813: Chayen and Bitensky, *Practical Histochemistry*, Chichester; New York: Wiley, 1991. The distinction between DAF-7 and DAF-2 regulation of longevity could also reflect a more profound regulation of metabolism by the DAF-2 pathway than the DAF-7 pathway (Fig. 4). For example, based on precedents from TGF- $\beta$  signaling in other systems and analysis of this pathway in *C. elegans*, all of the known signaling output of the DAF-7 TGF- $\beta$  pathway are via downstream Smad transcriptional regulation (infra). Insulin signaling, and by extension, DAF-2 signaling, is more ramified: outputs from this receptor regulate sugar transport, metabolic enzyme activities, translation of mRNAs encoding these and other enzymes, as well as transcription (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994).

We suggest that it is the regulatory output distinct to the DAF-2 pathway that controls longevity. Alternatively, TGF- $\beta$  and insulin-like signals may converge only during the L1 stage, when diapause is regulated, and that after this stage, only DAF-2 signaling is necessary for normal metabolic control.

The involvement of insulin and TGF- $\beta$  signaling in *C. elegans* diapause control suggests that the homologous human pathways may similarly mediate response to famine. Just as environmental extremes can select for variation in the genetic pathways that regulate *C. elegans* dauer formation, famines and droughts in human history may have selected for analogous variants in the human homolog of the *daf* genes. In fact, heterozygous mice carrying either the *db* or *ob* recessive

diabetes genes, survive fasting about 20% longer than wild type controls (Coleman, *Science* 203: 663-665, 1979). The high frequency of Type II diabetes in many human populations may be the legacy of such selections.

**The DAF-3 Smad Protein Antagonizes DAF-7 TGF- $\beta$  Receptor Signaling in the *C. elegans* Dauer Regulatory Pathway**

In response to environmental signals *C. elegans* arrests development at the anatomically and metabolically distinctive third-larval dauer stage (Riddle In: *C. elegans* N, D.L. Riddle, T. Blumenthal, B.J. Meyer, J.R. Priess, eds., *Cold Spring Harbor Press*, 1997, pp. 739-768). Pheromone signal is transduced by chemosensory neurons (Bargmann and Horvitz, *Science* 251:1243, 1991) which couple to a TGF- $\beta$  signaling pathway (Ren et al., *Science* 274:1389, 1996; Schackwitz et al., *Neuron* 17:719, 1989), as well as an insulin-related signaling pathway (as discussed, *infra*) to trigger changes in the development of the many tissues remodeled in dauer larvae (Riddle, *supra*). Mutations in *daf-7* (a TGF- $\beta$  homolog (Estevez et al., *Nature* 365:644, 1993)), *daf-4* (a type II TGF- $\beta$  receptor (Estevez et al., *Nature* 365:644, 1993)), *daf-1* (a type I TGF- $\beta$  receptor), *daf-8*, and *daf-14* (Smad homolog) cause constitutive arrest at the dauer stage even in the absence of pheromone. These genes constitute a neuroendocrine signaling pathway that is active during non-dauer development: the DAF-7 TGF- $\beta$  signal is produced by the sensory neuron ASI during nondauer development, whereas *daf-7* expression in this neuron is inhibited during dauer-inducing conditions (Ren, *supra*).

*daf-7* and its receptors and Smad proteins are antagonists to *daf-3*. The dauer constitute phenotypes of mutations in the *daf-7* signal transduction pathway

genes (including putative null mutations) are fully suppressed by mutations in *daf-3*. These genetic data indicate that in the absence of *daf-7* signaling, *daf-3* acts to induce dauer arrest.

To discern the molecular basis of the DAF-3 function in this pathway, we determined the sequence and expression pattern of *daf-3*. Cosmids in the *daf-3* genetic region were assayed for gene activity by transformation. Cosmid B0217 partially complemented a *daf-3* mutation, while other cosmids from the region did not (Fig. 5A). A subclone of B0217 containing only the Smad homolog, but no other coding regions also rescued *daf-3*. Our detection of mutations in the Smad homolog (see below) confirmed its assignment to *daf-3*. Analysis of *daf-3* cDNAs revealed that the gene was transcribed from fifteen exons and was alternatively spliced upstream of the region conserved in Smad proteins. (Fig. 5B) The biological activity of these alternatively spliced isoforms is unknown. The nucleotide (SEQ ID NO: 11) and amino acid sequences (SEQ ID NO: 12) of DAF-3 are shown in Figs. 11 and 12, respectively.

Thus far, the *C. elegans* DAF-3 Smad protein is most closely related in sequence to DPC4, which is a putative cofactor for Smad1, Smad2, and Smad3 (Zhang et al., *Nature*, 383:168, 1996; Lagna et al., *Nature*, 383:832, 1996; Savage et al., *Proc.Natl.Acad.Sci.*, 93:790, 1996; Hahn et al., *Science*, 271:350 (1996). Smads have two conserved domains (Wrana et al., *Trends Genet.*, 12:493, 1996). DAF-3 has these two domains; compared to its closest known relative DPC-4, *daf-3* has 55% amino acid identity in domain I and 30% in domain II (Fig. 5C). However, DPC-4 is not the mammalian DAF-3 homologue: *C. elegans* Sma-4, for example, is more closely related to DPC-4 than DAF-3.

We identified three mutations in *daf-3*, all of which were isolated as

suppressors of *daf-7(e1372)*. *mgDf90* is a homozygous viable deletion of 15-90 kb  
 that removes the entire Smad gene (Fig. 5A). *mgDf90* was identified as a  
 spontaneous mutation that suppressed *daf-7* in the strain of GR1300 (*daf-7 (e1372)*  
 111; *mut-6(st 702) unc-22 (St192) IV*). Thus, suppression of the *daf-7* dauer  
 5 constitutive phenotype of *daf-3* is *daf-3* null phenotype, demonstrating that  
 wild-type DAF-3 acts antagonistically to signaling from the DAF-7 TGF- $\beta$   
 pathway signaling. *daf-3(mg125)* and *daf-3(mg132)* are missense mutations that  
 alter conserved residues in domains 1 and 2 respectively (Fig. 5C). Most of the  
 mutations detected in other Smads localize to a 45 amino acid segment of domain  
 10 II (Wrana et al., *Trends in Genet.* 12:493, 1996). Clustering of mutations is  
 observed even in DPC4, for which homozygous null mutations have been  
 identified (Hahn et al., *Science* 271:350, 1996), so the clustering is unlikely to be  
 due to selection for non-null mutations. This hotspot region was sequenced in nine  
*daf-3* alleles, and no mutations were detected. This difference in mutation location  
 15 may be a simple statistical anomaly, or may indicate functional differences  
 between DAF-3 and other Smad proteins, consistent with the fact that DAF-3 is  
 antagonized, rather than activated, by an upstream TGF- $\beta$  molecule.

To determine where DAF-3 may function in control of dauer formation,  
 we examined the expression pattern of a functional *daf-3*/Green Fluorescent  
 20 Protein (GFP) fusion gene. This was accomplished by replacing a *AvrII/SacI*  
 fragment from pGP8 with a PCR product in which several restriction sites were  
 inserted after the last codon of *daf-3* before the stop codon. A GFP/*unc-54* 3' end  
 PCR product from pPD95.81 was cloned into the 3' restriction sites to produce  
 pGP19. This DAF-3/GFP fusion partially rescues a *daf-3* mutant (Fig. 7). GFP  
 25 fluorescence therefore indicates the functional location of DAF-3. DAF-7

signaling from the ASI neuron begins during the L1 stage, and neuron ablations and dauer-formation assays in various environmental conditions indicate that the signal for dauer formation is also received during the first two larval stages (Ren et al., *Science* 274:1389, 1996, Schackwitz et al., *Neuron* 17:719, 1996; Bargmann and Horvitz, *Science* 251:1243, 1991; Golden and Riddle, *Developmental Biology* 102:368, 1984; Swanson and Riddle, *Developmental Biology* 84:27, 1981).

Therefore, we most extensively examined L1 larvae.

Almost every transgenic animal showed strong *daf-3*/GFP expression in head neurons (Fig. 6A), the ventral nerve cord (both cell bodies and processes, see Fig. 6B), the intestinal cells (Fig. 6C), especially the membrane adjacent to the intestinal lumen, the tail hypodermis, and tail neurons. For all GFP scoring, animals were grown at 25-26°C. For scoring of DAF-3/GFP in wild-type and in dauer constitutive mutant backgrounds, three or more lines were scored in each case. A large number of animals were surveyed to determine the expression pattern, and at least 30 animals were scored head-to-tail, and expression was tallied for each tissue. About half of the transgenic animals have weak expression in V blast cells, P blast cells, hyp7 hypodermal cells, and the pharynx. The weak expression impedes cell identification, but the main body of the pharynx is filled, implying expression in pharyngeal muscle (Fig. 6A). Expression is rarely detected in dorsal body wall muscle. The expression pattern in older larvae and adults is similar to that of L1 animals. In addition, DAF-3/GFP is expressed in the distal tip cells and in their precursors, Z1.a and Z4.p, throughout development (Fig. 6D, Fig. 8). DAF-3/GFP is also strongly expressed in unidentified vulval cells. In wild-type embryos of 200-400 cells, DAF-3/GFP is expressed uniformly throughout the embryo (Fig. 6E). Under the conditions of the experiment, which

promote reproductive growth, the subcellular localization of the DAF-3/GFP protein is mainly cytoplasmic (Fig. 6B-E, and see below).

Because DAF-3 activity may be regulated by the DAF-1 and DAF-4 TGF- $\beta$  receptors, we examined the expression of a DAF-4/GFP fusion in wild-type (Figs. 6A-6G). This construct complements a *daf-4* mutant. A 10 kb Sall fragment from cosmid CO5D2 contains 3 kb of sequence upstream of the *daf-4* transcriptional start, and all of the *daf-4* coding region except codons for the last fourteen residues of *daf-4*. This fragment was subcloned into the Sall site of the GFP plasmid TU#61 (Chalfie et al., *Science* 263: 802-805, 1994). This plasmid was injected into the *daf-4(m72)* strain to test the fusion for DAF-4 activity. More than 95% of the transgenic animals were rescued for the dauer-constitutive and small phenotypes of *daf-4(m72)*, indicating that the fusion has robust DAF-4 activity. The pattern of DAF-4/GFP expression is similar to that of *daf-3*/GFP, except that DAF-4/GFP is localized to membranes, consistent with its role as a receptor. DAF-4/GFP is expressed more strongly in the pharynx (Figs. 6F-G), and more weakly in the ventral nerve cord cell bodies and the body hypodermis. Expression of DAF-4/GFP in wild-type animals is detected later than DAF-3/GFP. DAF-4/GFP is first detectable at late embryogenesis when the embryo resembles an L1 larva. The DAF-4/GFP construct contains an older version of GFP than in DAF-3/GFP; in the older version, the chromophore takes longer to mature. To verify that the difference in embryonic expression of DAF-4/GFP and DAF-3/GFP is not an artefact of the slower maturation time in the *daf-4* strain, we used anti-GFP antibodies to assay GFP. These antibodies should recognize the two forms of GFP equally well. We found that the antibodies recapitulated the results with direct GFP fluorescence: DAF-3/GFP is expressed in early embryos; DAF-4/GFP

is not. DAF-4/GFP is also not expressed in membrane surrounding the intestinal lumen, unlike DAF-3/GFP.

The combination of the DAF-3 and DAF-4 expression patterns suggests that these genes act in target tissues to transduce pheromone-regulated DAF-7 neuroendocrine signals. The early expression of DAF-3 in embryos is also consistent with a model that DAF-3 acts during embryonic development, for example, to mediate the development of neuronal pathways that emit neuroendocrine signals that antagonize DAF-7 TGF- $\beta$  signaling during the L1 stage. However our data indicates that DAF-3 functions in transducing environmental signals during the L1 and L2 stages. This is supported by the following observations. (1) DAF-7 TGF- $\beta$  signal from ASI neurons occurs during the L1 and L2 stages and is repressed by dauer-inducing environmental conditions. (2) Expression of the DAF-4 type II receptor begins in very late embryogenesis. (3) Expression patterns of DAF-3 and DAF-4 are coincident in most of the tissues remodeled during dauer morphogenesis. For example, the cuticle secreted by the hypodermis is modified, the pharynx is slimmed, and the lumen of the intestine is less convoluted. In addition, somatic gonad development is arrested in dauers, and the distal tip cell, in which DAF-3 is expressed, is an important regulator of that development (Kimble, *Developmental Biology* 87:286, 1981). In addition, the intestine and hypodermis of dauer larvae contain large fat stores indicative of a metabolic shift to fat storage. The expression of both the DAF-4 TGF- $\beta$  family receptor kinase and the DAF-3 Smad protein in these target tissues is consistent with a model that the DAF-7 neuroendocrine signal from the ASI neuron is received directly by these tissues during non dauer development. In addition, the observation that DAF-4 and DAF-3 are expressed in many of the same cells is

consistent with a model that DAF-4 signaling to downstream Smads (DAF-8 and DAF-14 are likely candidates) directly regulates DAF-3 gene activity. The TGF- $\beta$  regulated nuclear localization and transcriptional activation of some Smad proteins suggests that DAF-3 might induce the dauer-specific changes by activating  
5 transcription in target tissues of genes required for dauer formation or repressing transcription of genes necessary for nondauer growth.

Smad1 and Smad2 relocate to become predominantly nuclear when the upstream TGF- $\beta$  signaling pathways are activated (Baker and Harland, *Genes and Development* 10: 1880, 1996; Hoodless et al., *Cell* 85:489, 1996; Liu et al.,  
10 *Nature* 381:620, 1996; Macias-Silva et al., *Cell* 87:1215, 1996). In wild-type, DAF-3/GFP is primarily, although not exclusively, cytoplasmic. DAF-3/GFP subcellular distribution was examined in head neurons in the vicinity of ASI (the cell that produces the DAF-7 signal), as well as in intestinal cells. DAF-3/GFP was predominantly cytoplasmic in all animals. However, in all animals, dim GFP  
15 fluorescence was observed in the nucleus of some of the cells with bright fluorescence, and in approximately twenty-five percent of the animals, equivalent DAF-3/GFP levels in the nucleus and cytoplasm has observed in one or more cells.

Because DAF-3 is antagonized by the other members of the DAF-7 TGF- $\beta$  pathway, we expect that DAF-3 is active (and perhaps localized to the  
20 nucleus) when these genes are inactive. We therefore observed the subcellular localization of the full-length DAF-3/GFP fusion protein in the head neurons, tail neurons, and intestine of dauer-constitutive mutant L1 worms, when DAF-3 gene activity is predicted to be highest. In DAF-1(*m402*), *daf-4(m72)*, *daf-7(m62)*, *daf-8(sa233)*, and *daf-14(m77)* mutants, DAF-3/GFP was predominantly  
25 cytoplasmic, although, as in wild-type, cells were seen with some GFP in the

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nucleus. In three *daf-4(m72)* mutant lines, DAF-3/GFP was localized to the nucleus more than in wild-type lines. When these strains were crossed to wild-type, the increased nuclear localization was seen in both the *daf-4* and wild-type segregants. Thus the increased nuclear GFP was a property of the array, rather than of *daf-4*. Even in the neurons nearest to ASI, where the DAF-7 signal should be strongest, no change in DAF-3/GFP subcellular localization was detected. The DAF-3/GFP fusion protein is predominantly cytoplasmic in L1 and L2 stages of larvae induced to form dauers by environmental conditions or by mutations in the insulin receptor pathway gene *daf-2*, rather than by mutations in the DAF-7 signaling pathway mutants (data not shown). The tissue-specific expression pattern of DAF-3/GFP was unaltered in these mutant backgrounds (data not shown).

The finding that DAF-3/GFP subcellular localization is not strongly responsive to DAF-7 signaling defects or to dauer-inducing environmental conditions does not rule out a role for DAF-3 in the nucleus in dauer formation. Even though we detect no change in DAF-3/GFP subcellular localization, we do detect some DAF-3/GFP in nuclei, and a minor change in nuclear localization or a change in activity due to phosphorylation state may couple DAF-3 to DAF-7 signaling. In fact, the subcellular localization of *Drosophila* MAD protein is not detectably altered in wild-type when receptor signaling to MAD occurs; relocalization is seen only if the DPP ligand is drastically overexpressed. It is unlikely that a set of undiscovered TGF- $\beta$  receptors regulates DAF-3. The *C. elegans* genome sequence is 90% complete, and there is only one candidate TGF- $\beta$  receptor gene other than *daf-1* and *daf-4*. If this receptor were a positive regulator of DAF-3, mutants would be expected to, like *daf-3* mutants, suppress

*daf-7* mutants. This receptor acts in a signaling pathway distinct from DAF-3, and it is not a suppressor of *daf-7*.

The implication from Smad homology that DAF-3 is active in the nucleus is supported by two additional observations. First, DAF-3/GFP is associated with chromosomes in intestinal cells during mitosis. These cells divide at the end of the L1 stage, and antibody staining with anti-GFP antibodies and anti- $\alpha$ -tubulin antibodies reveals that DAF-3/GFP is found associated with DNA between the spindles during mitosis (Fig. 8A). We see DAF-3 GFP co-localized with DAPI from prophase to late anaphase. DAF-3/GFP was associated with nuclei in prophase by the following criteria. The spindles were present on either side of the nucleus, but the nucleus has not completely broken down. In particular, an indistinct nucleolus was present. DAF-3/GFP continues to co-localize with DAPI until the chromosomes have separated to the normal distance by which nuclei are separated in the intestine, implying continued association until telophase. At this point in mitosis, DAF-3/GFP fades and becomes undetectable before the nuclei reform the nuclear envelope and nucleolus. Thus, DAF-3 can, indirectly or directly, bind DNA, consistent with the hypothesis that it is a transcriptional activator that acts in the nucleus. DAF-3 is not predicted from its mutant phenotype to have a role in mitosis. It is possible that the brighter GFP on mitotic chromosomes is due to increased access to DNA due to the breakdown of the nuclear envelope. The second indication of DAF-3 function in the nucleus is our examination of a truncated DAF-3/GFP fusion that is missing most of conserved domain II. The truncated construct pGP7 consists of 8 kb of *daf-3* fused to GFP. An 8 kb EcoR1 fragment from B0217 was cloned into the EcoR1 site of pBluescript SK(-). A PvuI/SalI fragment of this subclone was ligated to a

PvuI/SalI fragment from the GFP vector pPD95.81. The resulting plasmid contains ~2.5 kb of sequence upstream of the 5'-most exon of *daf-3* and coding region through the first 58 amino acid residues of domain II. The remaining 175 amino acids of *daf-3* and the 3' noncoding region are replaced with GFP and the *unc-54* 3' end. Three transgenic lines were isolated, and all had a similar phenotype. This fusion protein interferes with dauer induction; like a *daf-3* loss-of-function mutant, it suppresses mutations in *daf-7* (Fig. 7). This truncated protein is predominantly nuclear, suggesting that it represses dauer formation by acting in the nucleus (Fig. 8B). This result implies that wild-type DAF-3 also has a function in the nucleus. The full-length DAF-3/GFP construct also suppresses mutations in *daf-7*, as does a full-length DAF-3 construct without GFP (Fig. 7). This suppression indicates that overexpression of DAF-3 in the cytoplasm has dominant-negative activity, perhaps due to interference with DAF-3 interactions with receptors or cofactors such as other Smads.

The constitutive nuclear localization of truncated DAF-3/GFP fusion gene missing part of domain II suggests that control of Smad localization is complex. A Smad2 construct containing only the conserved domain II of the protein is constitutively nuclear, leading to the suggestion that the C-terminus is an effector domain, and the N-terminus tethers the protein in the cytoplasm (Baker and Harland, *Genes and Development* 10:1880, 1996; Hoodless et al., *Cell* 85:489, 1996; Liu et al., *Nature* 381:620, 1996; and Macias-Silva et al., *Cell* 87:1215, 1996). Our construct, in which the N-terminus is intact, is nuclear. Perhaps both domains provide tethering in the cytoplasm, and any disruption leads to nuclear entry. Alternatively, entry may be differently regulated for DAF-3 and Smad2. Significantly, Smad2, like Smad1 and Smad3 has an SSXS motif at the C terminus

(Zhang et al., *Nature* 383:168, 1996; Lagna et al., *Nature* 383:832, 1996; Savage et al., *PNAS* 93:790; Baker and Harland, *Genes and Development* 10:1880, 1996; Hoodless et al., *Cell* 85:489, 1996; Liu et al., *Nature* 381:620, 1996; Macias-Silva et al., *Cell* 87:1215, 1996; and Graf et al., *Cell* 85:479, 1996); this motif is a  
5 substrate for phosphorylation and required for nuclear localization of Smad2 (Baker and Harland, *Genes and Development* 10:1880, 1996; Hoodless et al., *Cell* 85:489, 1996; Liu et al., *Nature* 381:620, 1996; and Macias-Silva et al., *Cell* 87:1215, 1996). DAF-3 has a single serine in the C terminal region, and DPC4 has no serines at this location.

10 We propose a model for the TGF- $\beta$  pathway in dauer formation (Figs. 9A-B). The DAF-7 TGF- $\beta$  ligand, which is produced by the ASI sensory neuron in conditions that induce reproductive organ (Ren et al., *Science* 274:1389, 1996; Schakwitz et al., *Neuron* 17:719, 1996), binds to the DAF-1/DAF-4 receptor kinases on target tissues. These receptor kinases then phosphorylate the Smads  
15 DAF-8 and/or DAF-14, analogous to the phosphorylation and activation of Smad1, Smad2, and Smad3 (Zhang et al., *Nature* 383:168, 1996; Lagna et al., *Nature* 383:832, 1996; Savage et al., *PNAS* 93:790, 1996). We propose that DAF-3 functions like its closest homolog, DPC4, which dimerizes with phosphorylated Smad1 and Smad2, even under conditions that do not lead to detectable DPC4  
20 phosphorylation (Zhang et al., *Nature* 383:168, 1996; Lagna et al., *Nature* 383:832, 1996; and Savage et al., *PNAS* 93:790). We suggest that DAF-3 forms dauer-inducing homodimers in the absence of DAF-7 signaling (Figs. 9A-B) that are disrupted when DAF-3 heterodimerizes with a phosphorylated DAF-8 and/or DAF-14 (Fig. 9B). Because *daf-8* and *daf-14* are only partially redundant (Riddle  
25 et al., *Nature* 290:668, 1981; Vowels and Thomas, *Genetics* 130:105, 1992; and

Thomas et al., *Genetics* 134:1105, 1993), each is likely to perform a unique function in dauer formation. Thus, DAF-3/DAF-8 dimers are proposed to have different activity from DAF-3/DAF-14. Perhaps each activates a subset of genes required for dauer formation. The formation of DAF-8/DAF-3 and/or DAF-14/DAF-3 heterodimers antagonizes dauer induction by the DAF-3/DAF-3 homodimer. A *daf-8(sa233); daf-14(m77); daf-3(mgDf90)* triple mutant can form some dauers in dauer-inducing conditions (data not shown); we suggest that activity of the Daf-2 pathway may induce dauer in this mutant background.

The dauer genetic pathway represents a neuroendocrine pathway for control of a diapause arrest and its associated shifts in metabolism and rates of senescence (Ren et al., *Science* 274:1389, 1996; Schackwitz et al., *Neuron* 17:719, 1996; and Georgi et al., *Cell* 61:635, 1990). Similarly, activins, members of the TGF- $\beta$  family, were originally identified based on their neuroendocrine regulatory activity, for example, in regulation of gonadotropin signaling (Vale et al., in *Peptide Growth Factors and Their Receptors*, Sporn and Roberts, Eds., Springer-Verlag, Heidelberg, 1990). The DAF-7 signal is not the only signal that is necessary for reproductive development. Because mutations in the DAF-7 TGF- $\beta$  pathway and in the DAF-2 insulin-like signaling pathway cause the same dauer arrest phenotypes, we propose that both the DAF-7 TGF- $\beta$  signals and the DAF-2 insulin-like signals are necessary for reproductive development. The involvement of an insulin-like signaling pathway in diapause with its associated metabolic shifts is consistent with metabolic regulation by insulin in vertebrates. Genetic experiments indicate that these pathways act in parallel (Riddle et al., *Nature* 290:668, 1981; Vowels and Thomas, *Genetics* 130:105, 1992; and Thomas et al., *Genetics* 134:1105, 1993). In particular, *daf-3* mutants efficiently suppress *daf-7*

mutants, but not *daf-2* mutants, and *daf-16* mutants efficiently suppress *daf-2* mutants, but poorly suppress *daf-7* mutants. It is not yet clear whether these two signaling pathways coverage on target tissues or in other regulatory (e.g., hormone secreting) cells. However, the expression of the DAF-7 receptor pathway genes and the DAF-16 gene in essentially all target tissues suggests that the TGF- $\beta$  and insulin pathways act there, and therefore that integration must occur there. Thus, we suggest in Figs. 9A and 9B that the DAF-2 pathway converges on DAF-3/DAF-8/DAF-1 Smad signaling to regulate metabolic gene expression in target tissues.

The integration of insulin-like and TGF- $\beta$  signals in metabolic control has important implications for the molecular basis of diabetes. For example, these converging pathways for dauer control suggest that in human metabolic control both a DAF-7-like signal and insulin may be necessary for full metabolic control. Thus, declines in signaling from the human homolog of DAF-7 could underlie the insulin resistance associated with Type II diabetes. In fact the dauer pheromone has been reported to be a fatty acid and to cause down-regulation of DAF-7 expression (Ren et al., supra). Thus pheromone regulation of metabolism may be related to mammalian obesity induced diabetes, and a human mutation in DAF-7 or its receptors is expected to contribute to a diabetic condition, just like mutations in the insulin receptor. In addition if obesity or age or both cause human DAF-7 to decline, e.g., under high leptin conditions, such a result would explain late onset/obesity related diabetes.

**Converging Transcriptional Outputs of the Insulin and DAF-7 Endocrine Signals**

Further support for the view that insulin-like and DAF-7 neuroendocrine signals regulate common transcriptional targets via the DAF-16 Forkhead protein and the DAF-8, DAF-14, and DAF-3 Smad proteins, respectively, comes from the following experiments. First, we have shown that the a 30 base element in the myosin 2 promoter, previously shown to bind to DAF-3 and be responsive to DAF-7 signaling, is also responsive to DAF-2 insulin like signaling (Okkema, Development, 1994, 120(8):2175-86). This element has the following sequence (SEQ ID NO: 210): TCTCGTTGTTTGCCGTCGGAT**GTCTGCC**. The bolded nucleotide positions are conserved in the *Xenopus* activin response element.

Specifically, a GFP fusion of this element (multimerized 6x) expresses 24 units of fluorescence in wild type, but less than 4 units in a *daf-4* TGF- $\beta$  signaling mutant or in a *daf-2* insulin- like signaling mutant. This repression of expression by lack of neuroendocrine input is relieved by mutations in *daf-3* in the case of the *daf-4* mutant and *daf-16* in the case of the *daf-2* mutant. The *daf-4*; *daf-3* double mutant expresses 12 units of GFP fluorescence and the *daf-2*; *daf-16* double mutant expresses 18 units of GFP fluorescence. These data strongly support the model that DAF-16 and DAF-3 bind to the same element in the myosin promoter. This is biologically relevant since the pharynx is smaller in dauer arrested animals, consistent with lower pharyngeal myosin expression in animals with defective DAF-7 or DAF-2 signaling.

### **Serotonergic Input to the Dauer Pathway**

We have further shown that mutants completely lacking in serotonin have defects in metabolic control. Specifically we have knocked out the serotonin synthesis gene, tryptophan hydroxylase, *cod-5*, by directed mutagenesis. *Cod-5* is

the aromatic amino acid hydroxylase that synthesises serotonin from the precursor L tryptophan (Fig. 42). It is the rate determining step in the synthesis of serotonin, and we have shown that it is only transcribed in the serotonergic neurons of *C. elegans*.

5           Our deletion mutant deletes most of the *cod-5* gene and causes a frameshift in the remaining coding region (Fig. 43). This mutant makes no serotonin as measured with antiserotonin antibody staining. The promoter of *cod-5* fused to GFP displays all of the serotonergic neurons of *C. elegans*, NSM, HSN, ADF, RIH (but not VC4 and VC5 which probably uptake 5HT from  
10   surrounding serotonergic neurons).

          The *cod-5* null mutant has a number of behavioral abnormalities, including egg laying defects, fertility declines, thermal regulation defects, and hyperactive movement, but most dramatic is that up to half of the mutant animals arrest at the dauer stage and accumulate large amounts of fat. This is quite similar  
15   to the regulation of feeding, appetite, and metabolism by serotonin in vertebrates. The behavior of *cod-5* mutants also shows the hallmarks of defects in DAF-7 signaling: the *cod-5* mutant animals tend to cluster at the edge of a lawn of bacteria, as if they are attracted to each other and repelled by the bacteria. This type of behavior is also seen in an NPY receptor mutant, *bor-1*. It is possible that  
20   DAF-7 normally regulates the secretion of the NPY like ligand of *bor-1*, and 5HT regulates DAF-7. This would explain the dauer arrest and bordering behavior of *cod-5* mutants, that it acts high in the pathway of DAF-7.

          5HT production is normally under feeding and temperature control: wild type *C. elegans* makes almost undetectable levels of 5HT when starved and  
25   makes lower amounts at low temperature. We believe that 5HT receptors are

expressed on particular regulatory neurons that also express or respond to the DAF-7 or DAF-2 signals, either as ligands or receptors. 5HT regulation of metabolism may occur via the DAF-7 pathway or the DAF-2 pathway, for example, by regulating expression of DAF-7, expression or secretion of the DAF-2 ligands, or signaling from the receptors. Moreover, given that *cod-5* mutations induce the same behavioral changes (that is, crowding at the edge of food) as *daf-7* mutants (in distinction from *daf-11* or *daf-2* pathway mutants), we believe that there is 5HT input to the *daf-7* pathway.

Our discovery of 5HT input to *C. elegans* metabolic control is important because it may reveal the mechanism by which drugs like dexfenfluramine and fluoxetine control weight in humans (Weiser et al., J Clin Pharmacol, 1997, 37(6):453-73). For example, if 5HT input to worm metabolic control is via the DAF-7 signaling system, the mechanism of action of serotonergic signals in metabolic control in mammals may be via serotonin modulation of expression or secretion of the mammalian DAF-7 homologue.

In addition, the *cod-5* promoter-GFP fusion is valuable for its ability to display serotonergic neurons, for example, for screens of mutants that fail to generate serotonergic neurons or screens for mutants that generate ectopic serotonergic neurons. Such a promoter fusion, for example, facilitates the identification of the neural pathway for the generation of 5HT neurons. In fact, the transcription factor *unc-86* has already been identified as part of that pathway. *Unc-86* mutants cause a lack of serotonin synthesis, due to loss of *cod-5* expression in all serotonergic neurons except ADF, and we have shown that the accumulation of serotonin in the NSM in an *unc-86* mutant is due to reuptake of 5HT, presumably from the ADF site of serotonin synthesis. Prozac, a reuptake

inhibitor, causes 5HT accumulation in the NSM to disappear in *unc-86* mutants.

### Cloning Mammalian DAF Sequences

Based on our isolation of novel nematode DAF cDNAs, the isolation of mammalian DAF nucleic acid sequences, including human DAF sequences, is made possible using the sequences described herein and standard techniques. In particular, using all or a portion of a nematode DAF sequence, one may readily design oligonucleotide probes, including degenerate oligonucleotide probes (i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be based upon the sequence of either strand of the DNA.

Exemplary probes or primers for isolating mammalian DAF sequences preferably correspond to conserved blocks of amino acids, for example, conserved DAF motifs. Exemplary motifs are as follows:

DAF-2 (tyrosine kinase domain) (SEQ ID NO: 33)

1242 KFHEWAAQICDGMAYLESLKFCHRDLAARNCMINRDETVKIGDFGM  
15 ARDLFYHDYYKPSGKRMMPVRWMSPELKDGGKFSKSDVWSFGVVLYE  
MVTLGAQPHYGLSNDEVLNYIGMARKVIKKPEC 1368

DAF-2 (ligand binding domain) (SEQ ID NO: 34)

242 NTTCCQKSCAYDRLLPTKEIGPGCDANGDRCHDQCVGGCERVNDATA  
CHACKNVYHKGKCKIEKCD AHL YLLLQRRCVTREQLQLNPVLSNKTVPK  
20 ATAGLCSDKCPDGYQINPDDHRECRKCVGKCEIVC 372

DAF-2 (67 amino acid motif) (SEQ ID NO: 79)

1158 AIKINVDDPASTENLNYLMEANIMKNFKTNFIVQLYGVISTVQPAMV

VMEMMDLGNLRDYLRSKRED 1224

DAF-2 (54 amino acid motif) (SEQ ID NO: 80)

1362 VIKKPECCENYWYKVMKMCWRYSPDRPTFLQLVHLLAAEASPEFR  
DLSFVLTD 1415

5 DAF-2 (69 amino acid motif) (SEQ ID NO: 81)

404 KQDSGMASELKDIFANIHTITGYLLVRQSSPFISLNMFRNLRRIEAKSL  
FRNLYAITVFENPNLKKLFD 472

DAF-2 (52 amino acid motif) (SEQ ID NO: 82)

98 FPHLREITGTLLVFETEGLVDLRKIFPNLRVIGGRSLIQHYALIYRN  
10 PDLE 149

DAF-2 (46 amino acid motif) (SEQ ID NO: 83)

149 EIGLDKLSVIRNGGVRIIDNRKLCYTKTIDWKHLITSSINDVVVDN 194

DAF-2 (36 amino acid motif) (SEQ ID NO: 84)

1112 YNADDWELRQDDVVLGQQCGEGSFGKVYLG TGNNVV 1147

15 DAF-3 (Smad Domain I) (SEQ ID NO: 35)

240 FDQKACESLVKKLKDKKNDLQNLIDVVL SKGTKYTGCITIPRTL DGR  
LQVHGRKGFP HVVYGKLWRFNEMTKNETRHVDHCKHAFEMKSDMVC  
VNPYHYEIVI 342

DAF-3 (Smad Domain II) (SEQ ID NO: 36)

20 690 NRYSLGLEPNPIREPVAFKVRKAIVD GIRFSYKKDGSVWLQNRMKYPV

FVTSGYLDEQSGGLKKDKVHKVYGCASIKTF 768

DAF-3 (79 amino acid motif) (SEQ ID NO: 85)

819 DSLAKYCCVRVSFCKGFGEAYPER 842

DAF-16 (forkhead DNA binding domain) (SEQ ID NO: 37)

5 727 KKT TTRNAWGNMSY AELITTAIMASPEKRLTLAQVYEW MVQNPY  
FRDKGDSNSSAGWKNSIRHNLSLHSR FMRIQNEGAGKSSWWVINPDAKPG  
MNPRRTRERS 1044

DAF-16 (103 amino acid motif) (SEQ ID NO: 54)

242 KKT TTRNAWGNMSY AELITTAIMASPEKRLTLAQVYEW MVQNPY  
10 FRDKGDSNSSAGWKNSIRHNLSLHSR FMRIQNEGAGKSSWWVINPDAKPG  
MNPRRTR 344

DAF-16 (41 amino acid motif) (SEQ ID NO: 55)

137 TFMNTPDDVMMNDDMEPIPRDRCNTWPMRRPQLEPPLNSSP 177

DAF-16 (109 amino acid motif) (SEQ ID NO: 56)

15 236 DDTVSGKKTTTTRNAWGNMSY AELITTAIMASPEKRLTLAQVYEW  
VQNPYFRDKGDSNSSAGWKNSIRHNLSLHSR FMRIQNEGAGKSSWWVI  
NPDAKPGMNPRRTR 344

DAF-16 (98 amino acid motif) (SEQ ID NO: 58)

372 KPNPWGEESYSDIIAKALESAPDGRLKLNEIYQWFS DNIPYFGERSSPE  
20 EAAGWKNSIRHNLSLHSR FMRIQNEGAGKSSWWVINPDAKPGMNP  
RRTR 469

Using such motifs, mammalian DAF-2, DAF-3, and DAF-16 genes may be isolated from sequence databases (for example, by the use of standard programs such as Pileup). Alternatively, such sequences may be used to design degenerate oligonucleotide probes to probe large genomic or cDNA libraries directly. General methods for designing and preparing such probes are provided, for example, in Ausubel et al., *Current Protocols in Molecular Biology*, 1996, Wiley & Sons, New York, NY; and *Guide to Molecular Cloning Techniques*, 1987, S. L. Berger and A. R. Kimmel, eds., Academic Press, New York. These oligonucleotides are useful for DAF gene isolation, either through their use as probes for hybridizing to DAF complementary sequences or as primers for various polymerase chain reaction (PCR) cloning strategies. If a PCR approach is utilized, the primers are optionally designed to allow cloning of the amplified product into a suitable vector. PCR is particularly useful for screening cDNA libraries from rare tissue types.

Hybridization techniques and procedures are well known to those skilled in the art and are described, for example, in Ausubel et al., *supra*, and *Guide to Molecular Cloning Techniques, supra*. If desired, a combination of different oligonucleotide probes may be used for the screening of the recombinant DNA library. The oligonucleotides are, for example, labelled with  $^{32}\text{P}$  using methods known in the art, and the detectably-labelled oligonucleotides are used to probe filter replicas from a recombinant DNA library. Recombinant DNA libraries (for example, human cDNA libraries, such as hypothalamus- or pancreas-derived cDNA libraries, particularly for DAF-2 and DAF-7 cDNAs) may be prepared according to methods well known in the art, for example, as described in Ausubel

et al., *supra*, or may be obtained from commercial sources.

For detection or isolation of closely related DAF sequences, high stringency hybridization conditions may be employed; such conditions include hybridization at about 42°C and about 50% formamide; a first wash at about  
5 65°C, about 2X SSC, and 1% SDS; followed by a second wash at about 65°C and about 0.1% SDS, 1X SSC. Lower stringency conditions for detecting DAF genes having less sequence identity to the nematode DAF genes described herein include, for example, hybridization at about 42°C in the absence of formamide; a first wash at about 42°C, about 6X SSC, and about 1% SDS; and a second wash at  
10 about 50°C, about 6X SSC, and about 1% SDS.

As discussed above, DAF-specific oligonucleotides may also be used as primers in PCR cloning strategies. Such PCR methods are well known in the art and are described, for example, in *PCR Technology*, H.A. Erlich, ed., Stockton Press, London, 1989; *PCR Protocols: A Guide to Methods and Applications*, M.A.  
15 Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, eds., Academic Press, Inc., New York, 1990; and Ausubel et al., *supra*. Again, sequences corresponding to conserved regions in a DAF sequence (for example, those regions described above) are preferred for use in isolating mammalian DAF sequences. Such probes may be used to screen cDNA as well as genomic DNA libraries.

20 Sequences obtained are then examined (for example, using the Pileup program) to identify those sequences having the highest amino acid sequence identity to the *C. elegans* sequence, particularly in or between conserved DAF domains (for example, those domains described above). In one particular example, the human FKHR, FKHL1, and AFX genes are 10<sup>33</sup> more closely related to the  
25 DAF-16 forkhead domain than the next most closely related forkhead domain

protein, making FKHR, FKHRL1, and AFX candidates for mammalian DAF-16 genes.

Following isolation of such candidate genes by sequence homology, the genes are then tested for their ability to functionally complement a *daf* mutation.

5 This is most readily assayed by transformation of the sequence into a *C. elegans* strain having an appropriate mutant background. Exemplary *C. elegans* transformation techniques are described, for example, in Mello et al., *EMBO J.* 10: 3959-3970, 1991, and assays for DAF-2, DAF-3, and DAF-16 polypeptide function are described herein. To be considered useful in the invention, a  
10 mammalian sequence need not fully complement a *C. elegans* defect, but must provide a detectable level of functional complementation.

The DAF, AGE, or AKT gene homologue identified as above, may also complement or alter the metabolic phenotypes of a mammalian cell line.

For example, addition of DAF-7, TGF- $\beta$ -like growth factor to an insulin  
15 responsive cell line (e.g., the 3T3-L1 cell line) may accentuate insulin responsiveness. Similarly genetic transformation of such a cell line with wild type or dominantly activated versions of a DAF, AGE, or AKT gene may alter metabolism. Such perturbations of metabolic control are stringent tests of candidate genes as DAF, AGE, or AKT homologues.

20 In addition, if that mammalian candidate homologue acts in a metabolic control pathway, and is expressed in similar metabolic control tissues (liver, adipose), it is likely to function homologously to DAF proteins from *C. elegans*. Addition of a wild type or activated DAF, AKT, or AGE protein (for example by VP16 activation of the DAF-3 or DAF-16 transcription factors) can confer on cell  
25 lines altered metabolic phenotypes. Thus supplying *daf*, *age*, or *akt* gene activity

to such a cell line can alter its metabolism. This is one exemplary test of homologous DAF function in metabolic control.

### **DAF Polypeptide Expression**

5 In general, DAF polypeptides according to the invention may be produced by transformation of a suitable host cell with all or part of DAF-encoding cDNA fragment (e.g., one of the cDNAs described herein or isolated as described above) in a suitable expression vehicle.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The DAF polypeptide may be produced in a prokaryotic host (e.g., E. coli) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae*, insect cells, e.g., Sf9 or Sf21 cells, or mammalian cells, e.g., COS 1, NIH 3T3, or HeLa cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; also, see, e.g., Ausubel et al., *supra*). The method of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*supra*); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (P.H. Pouwels et al., 1985, Supp. 1987).

One preferred expression system is the baculovirus system (using, for example, Sf9 cells and the method of Ausubel et al., *supra*). Another baculovirus system makes use of the vector pBacPAK9 and is available from Clontech (Palo Alto, CA).

Alternatively, an DAF polypeptide is produced in a mammalian system, for example, by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (*supra*); methods for constructing such cell lines are also

5 publicly available, e.g., in Ausubel et al. (*supra*). In one example, cDNA encoding the DAF protein is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the DAF protein-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300  $\mu$ M methotrexate in the cell culture medium (as described in

10 Ausubel et al., *supra*). This dominant selection may be accomplished in most cell types. Recombinant protein expression may be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (*supra*); such methods generally involve extended culture in medium containing gradually increasing

15 levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., *supra*). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR<sup>-</sup> cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line

20 or DHFR-mediated gene amplification.

In yet other alternative approaches, the DAF polypeptide is produced *in vivo* or, preferably, *in vitro* using a T7 system (see, for example, Ausubel et al., *supra*, or other standard techniques).

Once the recombinant DAF protein is expressed, it is isolated, e.g.,

25 using affinity chromatography. In one example, an anti-DAF protein antibody

(e.g., produced as described herein) may be attached to a column and used to isolate the DAF protein. Lysis and fractionation of DAF protein-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., *supra*).

5           Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, eds., Work and Burdon, Elsevier, 1980).

10           Polypeptides of the invention, particularly short DAF polypeptide fragments, may also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

15           These general techniques of polypeptide expression and purification may also be used to produce and isolate useful DAF fragments or analogs (described herein).

### **Anti-DAF Antibodies**

20           Using any of the DAF polypeptides described herein or isolated as described above, anti-DAF antibodies may be produced by any standard technique. In one particular example, a DAF cDNA or cDNA fragment encoding a conserved DAF domain is fused to GST, and the fusion protein produced in E. coli by standard techniques. The fusion protein is then purified on a glutathione column, also by standard techniques, and is used to immunize rabbits. The antisera obtained is then itself purified on a GST-DAF affinity column, for example, by the method of Finney and Ruvkun (*Cell* 63:895-905, 1990), and is shown to

specifically identify GST-DAF, for example, by Western blotting.

Polypeptides for antibody production may be produced by recombinant or peptide synthetic techniques (see, e.g., *Solid Phase Peptide Synthesis*, *supra*; Ausubel et al., *supra*).

5 For polyclonal antisera, the peptides may, if desired, be coupled to a carrier protein, such as KLH as described in Ausubel et al, *supra*. The KLH-peptide is mixed with Freund's adjuvant and injected into guinea pigs, rats, or preferably rabbits. Antibodies may be purified by any method of peptide antigen affinity chromatography.

10 Alternatively, monoclonal antibodies may be prepared using a DAF polypeptide (or immunogenic fragment or analog) and standard hybridoma technology (see, e.g., Kohler et al., *Nature* 256:495, 1975; Kohler et al., *Eur. J. Immunol.* 6:511, 1976; Kohler et al., *Eur. J. Immunol.* 6:292, 1976; Hammerling et al., *In Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, NY, 1981; Ausubel et al., *supra*).

15 Once produced, polyclonal or monoclonal antibodies are tested for specific DAF recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., *supra*). Antibodies which specifically recognize a DAF polypeptide described herein are considered to be useful in the invention. Anti-DAF antibodies, as isolated above, may be used, e.g., in an immunoassay to measure or monitor the level of DAF polypeptide produced by a mammal or to screen for compounds which modulate DAF polypeptide production (for example, in the screens described herein). In one particular example, antibodies to human DAF-7 polypeptide are useful for screening blood samples  
25 from patients to determine whether they possess decreased DAF-7 polypeptide

levels. Such antibodies may be used in any immunological assay, for example, an ELISA assay, and a decrease in DAF-7 is taken as an indication of a diabetic condition, for example, obesity onset Type II diabetes. In another particular example, anti-DAF antibodies are useful for carrying out pedigree analysis. For example, blood samples from individuals may be screened with anti-DAF-7 antibodies to detect those members of a family with a predisposition to a diabetic condition. Anti-DAF antibodies may also be used to identify cells that express a DAF gene.

#### **DAF-7 Therapy for Obesity-Onset Type II Diabetes**

Our data indicates that DAF-7 represents an endocrine hormone for metabolic control that acts synergistically with insulin. Declines in DAF-7 may be induced by obesity, just as the dauer pheromone, a fatty acid, causes declines in *C. elegans* DAF-7 production.

Accordingly, obesity onset Type II diabetes, glucose intolerance, and the associated atherosclerosis may be treated if DAF-7 hormone is injected intramuscularly or intravenously (Fig. 23).

In addition, antibodies to human DAF-7 should detect declines in DAF-7 in pre-diabetic, glucose-intolerant, or obesity induced diabetes. Such antibodies will detect DAF-7 levels in blood, just as insulin levels are detected in metabolic disease.

DAF-7 therapeutic potential and dosage can be developed in mouse models of obesity onset diabetes, for example, the db and ob mouse.

DAF-7 may be injected either intravenously or intramuscularly, in analogy to insulin therapy.

The decision of which classes of diabetics to treat with DAF-7 will come from a combination of blood tests for DAF-7 levels and genetic testing to determine which *daf*, *age*, or *akt* mutations a particular diabetic or pre-diabetic patient carries.

5    **Screening Systems for Identifying Therapeutics**

Based on our experimental results, we have developed a number of screening procedures for identifying therapeutic compounds (e.g., anti-diabetic and anti-obesity pharmaceuticals or both) which can be used in human patients. In particular examples, compounds that down regulate *daf-3* or *daf-16* or their human  
10 homologs are considered useful in the invention. Similarly, compounds that up regulate or activate *daf-1*, *daf-2*, *daf-4*, *daf-7*, *daf-8*, *daf-11*, *daf-14*, *age-1*, or *akt* (or each of their corresponding human homologs) are also considered useful as drugs for the treatment of impaired glucose tolerance conditions, such as diabetes and obesity. In general, the screening methods of the invention involve screening  
15 any number of compounds for therapeutically active agents by employing any number of *in vitro* or *in vivo* experimental systems. Exemplary methods useful for the identification of such compounds are detailed below.

The methods of the invention simplify the evaluation, identification, and development of active agents for the treatment and prevention of impaired glucose  
20 tolerance conditions, such as diabetes and obesity. In general, the screening methods provide a facile means for selecting natural product extracts or compounds of interest from a large population which are further evaluated and condensed to a few active and selective materials. Constituents of this pool are then purified and evaluated in the methods of the invention to determine their anti-

diabetic or anti-obesity activities or both.

Below we describe screening methods for evaluating the efficacy of a compound as anti-diabetic or anti-obesity agents or both. These examples are intended to illustrate, not limit, the scope of the claimed invention.

## 5 Test Extracts and Compounds

In general, novel drugs for the treatment of impaired glucose tolerance conditions are identified from large libraries of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will  
10 understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation  
15 broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from  
20 Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge,

MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

5 In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their anti-diabetic and anti-obesity activities should be employed whenever possible.

10 When a crude extract is found to have anti-diabetic or anti-obesity activities or both, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having anti-diabetic  
15 or anti-obesity activities. The same *in vivo* and *in vitro* assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for the treatment of pathogenicity are  
20 chemically modified according to methods known in the art. Compounds identified as being of therapeutic value are subsequently analyzed using any standard animal model of diabetes or obesity known in the art.

There now follow examples of high-throughput systems useful for evaluating the efficacy of a molecule or compound in treating (or preventing) an  
25 impaired glucose tolerance condition.

### Nematode Release of Dauer Arrest Bioassays

To enable mass screening of large quantities of natural products, extracts, or test compounds in an efficient and systematic fashion, *C. elegans* mutant dauer larvae (e.g., *C. elegans* containing mutations described herein, such as *C. elegans daf-2* mutant dauer larvae) are cultured in wells of a microtiter plate, facilitating the semiautomation of manipulations and full automation of data collection. In one particular example, the assay for dauer release involves a measurement of culture turbidity. Specifically, dauer larvae are treated with candidate compounds and allowed to incubate. If dauer release occurs, the animals grow and reproduce, and consume their light-scattering bacterial food source, decreasing the turbidity of the microtiter well culture. Thus, dauer release is measured by the extent of the decrease in culture turbidity. This type of assay allows millions of microtiter samples to be simultaneously screened.

As discussed above, compounds that down regulate DAF-3 or DAF-16 activities or up regulate DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT activities are considered useful in the invention. Such compounds are identified by their effect on dauer formation in *C. elegans* strains carrying mutations in these genes (as described above).

In particular examples, nematodes bearing mutations in the DAF-2 polypeptide arrest as dauer larvae, never producing progeny. All of the metabolic and growth arrest phenotypes caused by lack of *daf-2* are suppressed by mutations in *daf-16*. Mutations in the PI 3-kinase, AGE-1, have the same phenotype as lack of *daf-2*, and such mutations are also suppressed by *daf-16* mutations. Biochemical analysis of insulin signaling in mammals supports the view that

AGE-1 transduces signals from the DAF-2 receptor by generating a PIP3 signal. Because *daf-16* mutations suppress lack of *daf-2*, or *age-1* gene activity, it is believed that PIP3 down regulates or modifies *daf-16* gene activity. The biochemical overlap between DAF-2/AGE-1 and insulin receptors/PI 3-kinase indicates that the human homolog of the *C. elegans daf-16* gene acts in the insulin pathway as well. Thus, the *C. elegans* insulin signaling pathway yields the surprising result that the animals can live without insulin signaling, provided they are mutant in *daf-16*. This analysis therefore indicates that a compound that inhibits DAF-16 activity would reverse the effects of diabetic lesions, e.g., in the production or secretion of insulin or in the reception of insulin signals by target tissues. Such drugs would be expected to be efficacious in the treatment of insulin deficiencies due to pancreatic  $\beta$  cell destruction in Type I diabetes, as well as some Type II diabetes due to defects in insulin signaling.

To evaluate the ability of a test compound or an extract to decrease *daf-16* gene activity, mutant *daf-2* (e1370); *daf-16* (mgDf50) animals carrying an integrated human DAF-16 gene are incubated in microtiter dishes in the presence of a test compound. This human DAF-16 gene supplies all of the DAF-16 activity in the *C. elegans* strain and thus allows *daf-2*-induced dauer arrest unless its activity is decreased by the candidate test compound. If desired, various concentrations of the test compound or extract can be inoculated to assess the dosage effect. Control wells are incubated in the absence of a test compound or extract. Plates are then incubated at 25°C. After an appropriate period of time, e.g., 2 to 5 days, wells are examined for progeny. The presence of progeny is taken as an indication that the test compound or extract is effective at inhibiting *daf-3* or *daf-16* activity, and therefore is considered useful in the invention. Any

compound that inhibits DAF-16 gene activity (or activates upstream signaling in the absence of receptor function) will allow reproduction. This is shown schematically in Fig. 19.

Alternatively, a diabetic condition may arise from defects in the DAF-7 TGF- $\beta$  signaling pathway. Since a decrease in DAF-3 activity bypasses the need for DAF-7 activity in *C. elegans* metabolic control, drugs that down regulate DAF-3 activity are useful for ameliorating the metabolic defects associated with diabetes. To screen for such drugs, *daf-7* (e1372); *daf-3* (mg90) nematodes expressing human DAF-3 are exposed to chemicals as described above. In this strain, human DAF-3 supplies all DAF-3 activity, causing *daf-7* induced dauer arrest unless its activity is inhibited (Fig. 20). Compounds capable of inhibiting this activity are considered useful therapeutics in the invention.

Finally, in a less complex screen for drugs that inhibit *C. elegans daf-3* or *daf-16*, *daf-7* or *daf-2* mutants are directly screened for compounds that decrease *C. elegans daf-3* or *daf-16* gene activity.

In addition, *C. elegans* worms carrying other *daf* mutations may be utilized in an assay to obtain additional information on the mode of action of the test compound in the insulin or TGF- $\beta$  signaling pathways. For example, a drug having PIP3 agonist activity would be expected to allow *age-1* and *daf-2* mutants (but not *akt* or *daf-7* mutants) to not arrest at the dauer stage. Similarly, drugs that inhibit *daf-3* are expected to suppress *daf-7* mutants but not *daf-2* or *age-1* mutants.

### **Exemplary Dauer Recovery Screen**

Using screens such as those described above, muscarinic agonists have

been shown to specifically promote dauer recovery in pheromone-induced dauers as well as particular classes of dauer constitutive mutants. Strikingly, the muscarinic agonists could not induce recovery of *daf-2* induced dauers, which have defective insulin-like signaling. This muscarinic pathway was also shown to regulate *A. caninum* recovery from dauer arrest. In mammals, such muscarinic agonists promote insulin release both *in vivo* and *in vitro* (Ahren et al., (1986) Diabetologia 29:827-836; and Miller (1981) Endocr. Rev. 2:471-494). We suggest that insulin-like secretory cells in the nematodes are regulated by cholinergic inputs in a metabolic control pathway that is homologous to the mammalian autonomic input to pancreatic beta cell activity. Drugs that activate cholinergic as well as other mammalian insulin release pathways may prove useful in the control of parasitic nematode life cycles. These experiments were carried out as follows.

#### **Strains and Growth Conditions**

All strains were maintained and handled as described in Brenner (1974) Genetics 77:71-94; and Sulston and Hodgkin (1988) Methods (Cold Spring Harbor Laboratory, Cold Spring Harbor. Animals were grown on standard NG agar plates. In this study, the mutations in *C. elegans* used were LGI: *daf-8(e1393)*; LGII: *daf-22(m130)*; LGIII: *daf-7(e1372)*, *daf-2(e1370)*, *daf-4(m63)*; and LGIV: *daf-1(m40)*, *daf-14(m77)*, *daf-10(e1387)*; LGX: *daf-12(m20)*. *Ancylostoma caninum* were maintained as described previously (Hawdon and Schad (1993) Exper. Parasitol. 77:489-491).

#### **Dauer Arrest Assay**

Minimal media plates were used for the drug assays: 3.0 g NaCl, 20 g agarose (Sigma-Type II #A6877) and 970 ml of water. The autoclaved solution was cooled to 50-55°C before 25 ml of 1M KPO<sub>4</sub> (pH 6.0), 1.0 ml 1M CaCl<sub>2</sub>, 1.0 ml of 1M MgSO<sub>4</sub>, and 1 ml of 5 mg/ml cholesterol were added. In some assays, 5 *Escherichia coli* (DH5α) bacteria arrested with streptomycin was added to each plate.

Animals were grown at 15°C for several generations and then were placed in a bleach solution to isolate eggs. 100-200 eggs were added to each 10 ml drug plate with food. In several assays, eggs were placed in 5-6 ml of S medium (Wood (1988) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)) 10 in a 15 ml polypropylene tube on a rotary platform at 25°C overnight for 12-16 hours without food. This yields a synchronous preparation of L1 animals. The synchronized L1s were placed onto the drug plates at 20°C. Two, four, and eight days later, plates were examined for the presence of arrested dauers and 15 reproductive non-dauers. When the non-dauers had reached the gravid adult hermaphrodite stage and were beginning to lay eggs, each plate was examined visually for the presence of dauers and non-dauers. Following this, animals from each plate were rinsed off the plate into a plastic dish containing 1% SDS (dauers are the only larval stage resistant to this treatment). After 30 minutes, dishes were 20 examined under the dissecting microscope for the presence of dauers and non-dauers.

### **Dauer Recovery Assay**

We found that the most effective assay for dauer recovery was to place dauer stage animals onto drug plates at 25°C without the addition of food. In

some experiments, 100-200 eggs or synchronized L1s were put onto the drug plates. For all experiments described herein, about 10,000 L1s were placed in 10 ml of S Medium containing 1-2 ml of a 0.4% (w/v) solution of *Escherichia coli* DH5 $\alpha$  bacteria in M9 solution (Wood (1988) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)) arrested with streptomycin, in a 25 ml flask on a rotating heated water bath at 25°C. For wild-type N2 dauers, 600  $\mu$ l of the 0.4% bacterial solution and pheromone was also added to flask as described in Gottlieb and Ruvkun (1994) Genetics 137:107120. The pheromone preparation is a solution prepared as follows. Animals were grown in a large flask for several generations, and then spun down. The supernatant was boiled down to a brownish powder and then ethanol extracted. After 72 hours of liquid growth, animals were centrifuged and the supernatant removed. Animals were then resuspended in a 15 ml tube with a pre-heated 25°C solution of 1% SDS and tubes were placed on a rocker at 25°C for 30 minutes. Animals were centrifuged and the SDS removed. Animals were washed with either M9 or S medium (Wood (1988) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)) 4-6 times. After a final spin, 100-200 dauers were placed onto the drug plates without food. 24 and 48 hours later, plates were scored for the number of dauers and non-dauer adults.

For each strain tested, a control plate without any drug, with and without food was also tested. With no drug, there was 40% recovery in N2 dauers. The high value for the control plate in N2 may have been due to experimental procedure. The background recovery rate for N2 was much higher than the background recovery rate for the dauer constitutive mutants where there was very little, if any, background recovery. The assay was performed at 25°C, which means that the *daf-c* mutants are still under full dauer inducing conditions.

However, for N2, no exogenous pheromone was added to the drug plate and therefore, even though the plates were kept at a high temperature and had no food, dauer-maintaining conditions may not have been as severe as for the *daf-c* mutants.

## 5                    *Drug Assay in A. caninum*

Hookworm infective L3 animals were collected from 1-4 wk old coproculture by the Baermann technique, and decontaminated with 1% HCL in BU buffer (50 mM Na<sub>2</sub>PO<sub>4</sub>/22 mM KH<sub>2</sub>PO<sub>4</sub>/70 mM NaCl, pH 6.8) (Hawdon and Schad (1991) in Developmental Adaptations in Nematodes., ed. C.A. Toft, A.A. a. L. B. (Oxford University Press, Oxford), pp. 274-298; and Hawdon and Schad (1991) J. Helm. Soc. Wash. 58:140-142) for 30 minutes at 22°C. Approximately 250 L3 animals were incubated in individual wells of a 96-well tissue culture plate containing 0.1 ml RPMI1640 tissue culture medium, supplemented with 0.25 mM HEPES pH 7.2, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml gentamycin, and 2.5 µg/ml amphotericin B. The L3 animals were activated to resume development and feeding by including 10% (v/v) canine serum and 25 mM S-methyl-glutathione (GSM; Hawdon et al. (1995) Exper. Parasitol. 80:205-211). Non-activated L3 animals were incubated in RPMI alone (i.e., without the stimulus). Stock solutions of the drugs were made in RPMI, and included in the incubation at the indicated concentrations. The agonists were tested for activation by incubation with the L3 animals in the absence of the normal stimulus (i.e., serum + GSM), whereas atropine was tested in the presence of the normal stimulus, as well as with the agonists. The L3 animals were incubated at 37°C 5% CO<sub>2</sub> for 24 hours. The percentage of feeding L3 animals was determined by

incubating the L3 animals with 2.5 mg/ml FITC-BSA for 2-3 hours, followed by counting the number of L3 animals that had ingested the labeled BSA by microscopic examination under epi-fluorescent illumination (Hawdon and Schad (1990) J. Parasit. 76:394-398). Each treatment was done in triplicate, and each  
5 experiment was repeated at least once.

### Neurotransmitter Regulation of Diapause

Dauer arrest is modulated by sensory inputs (Golden and Riddle (1984) Developmental Biology 102:368-378). Arrest at the dauer stage is controlled by parallel TGF- $\beta$  and insulin-like signaling pathways (Riddle (1988) in The  
10 Nematode Caenorhabditis elegans, ed. Wood, W.B. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), pp. 393-412; Riddle and Albert (1997) in C. elegans II, eds. Riddle, D.L., Blumenthal, T., Meyer, B.J. & Priess, J.R. (Cold Spring Harbor Laboratory Press), pp. 739-768; Thomas (1993) Bioessays 15:791-797; Riddle et al. (1981) Nature 290:668-671; Vowels and  
15 Thomas (1992) Genetics 130:105-123; Thomas et al. (1993) Genetics 134:1105-1117; Gottlieb and Ruvkun (1994) Genetics 137:107120; Georgi et al. (1990) Cell 61:635-645; Estevez et al. (1993) Nature 365:644-649; Ren et al. (1996) Science 274:1389-1391; Kimura et al. (1997) Science 277:942-946; and Morris et al. (1996) Nature 382:536-539.41). Animals arrest at the dauer stage when they are  
20 lacking signaling from either of these two pathways (Georgi et al. (1990) Cell 61:635-645; Estevez et al. (1993) Nature 365:644-649; Ren et al. (1996) Science 274:1389-1391; Kimura et al. (1997) Science 277:942-946; and Morris et al. (1996) Nature 382:536-539.41). Animals will arrest development at the dauer stage when high levels of pheromone result in the absence of both the

DAF-7/TGF- $\beta$  ligand which is secreted from the ASI sensory neuron as well as an as yet unidentified secretory cell that releases the insulin-like ligand. Lack of the TGF- $\beta$  ligand results in an upregulation of the DAF-3 Smad protein, while lack of the insulin-like ligand causes an upregulation of the DAF-16 forkhead

5 transcription factor. Therefore, for dauer arrest, two separate signaling pathways are involved. Recovery from the dauer arrest when pheromone levels decline is thought to involve up-regulation of these TGF- $\beta$  and insulin-like signals.

To detect possible neural inputs to this neuroendocrine system, we tested drugs that affect a variety of neurotransmitter signaling pathways, including  
10 agonists, antagonists, and reuptake inhibitors, for effects on either dauer arrest or dauer recovery. We have shown that muscarinic agonists (oxotremorine, arecoline, pilocarpine and muscarine) promoted dauer recovery. None of the drugs tested promoted dauer arrest under replete conditions.

### **Cholinergic Input to Dauer Recovery**

15 We tested drugs that affected the following mammalian neuronal pathways: adrenergic/noradrenergic, serotonergic, cholinergic, glutaminergic, dopaminergic, gabaergic and opiod for effects on *C. elegans* dauer induction and dauer recovery. In each category both agonists and antagonists were examined. Most drugs tested did not affect dauer recovery and the animals remained arrested  
20 at the dauer stage. However, multiple unrelated muscarinic agonists could promote dauer recovery. Four muscarinic agonists, oxotremorine, pilocarpine, arecoline, and carbachol (Avery et al. (1993) Genetics 134:455-464), promoted recovery of dauers induced by mutation as well as pheromone. The dose response curves in Figures 44A-44C show the muscarinic agonists induced about 50%

recovery of dauers induced by defective TGF- $\beta$  signaling in the *daf-7(e1372)* mutant, with a defect in the TGF- $\beta$  ligand. Similar results were seen with other mutants in the TGF- $\beta$  signaling pathway, *daf-1(m40)* in the type I TGF- $\beta$  receptor and *daf-4(m63)* in the type II TGF- $\beta$  receptor. For example, 30% of *daf-1(m40)* dauers recover in oxotremorine, whereas plates with no drug had less than 5% recovery. Similarly, 50% of *daf-4(m63)* dauers recover in oxotremorine, while plates with no drug had less than 1% recovery.

The infective "dauer" L3 of the hookworm *A. caninum* can be stimulated to resume feeding and development in vitro by incubation with canine serum and S-methyl-glutathione (GSM), but not by tissue culture medium alone (Sulston and Hodgkin (1988) Methods (Cold Spring Harbor Laboratory, Cold Spring Harbor)). However, when *A. caninum* L3 were incubated with either oxotremorine or arecoline in the tissue culture medium, 60-80% of the animals recovered, as indicated by the resumption of feeding. Therefore, muscarinic agonists mimicked the recovery induced by serum and GSM.

Figures 44A-44C show the dose response curves of two of the muscarinic agonists tested: oxotremorine and arecoline. In each figure we show the dose response for wild-type induced dauers, *daf-7(e1372)*, *daf-2(e1370)* and *A. caninum* dauers. Pilocarpine (data not shown) and oxotremorine (Figure 44A) induced maximum recovery of *daf-7(e1372)* dauers at 5 mM concentration, while wild-type pheromone-induced dauers reached maximum recovery at 1 mM. *A. caninum* L3 dauers also reached maximum recovery at 5 mM oxotremorine (Figure 44A), but failed to recover when incubated with pilocarpine. The maximal response for arecoline was 10 fold lower than for the other agonists in both *C. elegans* and *A. caninum* (Figure 44B). Concentrations of 1 mM to 5 mM of a drug

are routinely used in drug assays in *C. elegans* (Hart et al. (1995) Nature 378:82-85.21; Horvitz et al. (1982) Science 216:1012-1014; Lewis et al. (1980) Genetics 95:905-928; Lewis et al. (1980) Neuroscience 5:967-989; Maricq et al. (1995) Nature 378:78-81; McIntire et al. (1993) Nature 364:334-337; McIntire et al. (1993) Nature 364:337-341; Schinkmann and Li (1992) J. Comp. Neurol. 316:251-260.51; and Avery et al. (1993) Genetics 134:455-464). The unusually high doses may be due to a cuticle permeability barrier.

While the muscarinic agonists were potent inducers of recovery in *daf-7* induced and pheromone-induced dauers, they did not induce recovery of a *daf-2* mutant with defects in the *C. elegans* homologue of the mammalian insulin receptor gene (Figure 44A-44C). Thus the muscarinic recovery pathway depends on insulin-like signaling. Atropine specifically inhibits dauer recovery

To determine the specificity of the muscarinic response, we added both oxotremorine, the agonist, and atropine, a muscarinic antagonist, to plates varying the concentration of antagonist to obtain a dose response shown in Figure 44C. In 1 mM oxotremorine, 40% of the *daf-7(el372)* dauers recovered. However, in combination with 1 mM atropine, 1 mM oxotremorine only induced 5% recovery; at 5 mM atropine, the 1 mM oxotremorine response was completely abolished. For wild-type N2 dauers, the results were almost identical (Figure 44C). This suggested that the drug-induced recovery was a specific muscarinic response, since in mammals atropine is only a muscarinic antagonist and did not interfere with nicotinic receptors (Lefkowitz et al. (1996) in Goodman and Gilman's The Pharmacological Basis of Therapeutics, eds. Hardman, J.G. & Limbird, L.E., McGraw Hill, pp. 105-139; and Brown and Taylor (1996) in Goodman and Gilman's The Pharmacological Basis of Therapeutics, eds. Hardman, J.G. &

Limbrid, L.E., McGraw Hill, pp. 141-160).

Atropine (0.5 mM) inhibited recovery of *A. caninum* L3 incubated with serum and GSM by 99.5%. Moreover, *A. caninum* L3 incubated with 0.5 mM arecoline and 1.0 mM atropine failed to recover (Figure 44C). These data indicate  
5 that recovery from arrest in hookworm L3 is also mediated by a muscarinic signal.

Atropine inhibits *C. elegans* dauer recovery induced by food signals. When killed bacteria are added to pheromone-induced dauers at 25°C, 99% of the animals recovered (Figure 45A). Without bacteria, no dauers recovered in the same time period (Figure 45A). However, only 21% of the dauer larvae recovered  
10 in bacterial food plus atropine (Figure 45A). Similarly, atropine (0.5 mM) inhibited recovery of *A. caninum* L3 incubated with serum and GSM by 99.5%.

Temperature is a potent inducer of dauer recovery in animals bearing mutations in the TGF- $\beta$  or insulin-like signaling pathways. For example, null mutations in *daf-7* are temperature sensitive, and recovery of both *daf-7* and  
15 *daf-2*-induced dauer larvae was stimulated by shift to 15°C (Figure 45A). Temperature downshift in the absence of food did not induce dauer recovery in either *daf-7* or *daf-2* mutants (Figure 45A) nor did bacterial food at 25°C allow non dauer development. However, temperature downshift and addition of food induced more than 75% recovery of both mutants. This recovery in both *daf-7* and  
20 *daf-2* mutants was inhibited by atropine (Figure 45A-45B).

We tested whether it was necessary to have functioning sensory neurons to mediate the muscarinic induced response. *daf-10* mutants have abnormal mechanocilia and irregular contours in the amphid sensilla. A *daf-7(e1372); daf-10(e1387)* double mutant gave a maximum response of 13% recovery with 1  
25 mM oxotremorine. This suggests that the amphid neurons are necessary to

meditate the muscarinic response. Alternatively, it is possible that the amphid defects do not allow the drug to enter the worm, if the drug indeed does penetrate the worm through these neurons.

We also examined whether exogenous application of neurotransmitters could mimic the dauer pheromone to induce dauer arrest. We tested these drugs for induction of dauer arrest in wild-type and *daf-22* mutants. *daf-22* is a mutant that does not secrete pheromone, but will arrest at the dauer stage when exposed to exogenous pheromone (Golden and Riddle (1985) Molecular and General Genetics 198:534-536). None of the drugs tested caused dauer entry under favorable growth conditions. The drugs were active in the plate because several of the drugs caused either paralysis, death, or egg-laying defects.

#### **Dauer Recovery by Muscarinic Agonists**

Arrest at the dauer stage is a nematode survival strategy that is a specific example of the related and phylogenetically general diapause arrest. In *C. elegans*, dauer arrest occurs under harsh environmental conditions whereas in the hookworm, *A. caninum*, a parasitic nematode, diapause is a non-conditional stage in the life cycle (Riddle and Bird (1985) J. Nematol. 17:165-168; and Schmidt and Roberts (1985) Foundations of Parasitology (Times Mirros/Mosby College Publishing)). Dauer recovery is regulated by levels of pheromone, food, and temperature in *C. elegans*, whereas in *A. caninum* unknown host factors induce dauer recovery upon infection (Golden and Riddle (1984) Developmental Biology 102:368-378).

We have shown that muscarinic agonists cause dauer recovery in both *C. elegans* and *A. caninum*, and that this recovery is specifically inhibited by the

muscarinic antagonist atropine. The endogenous neurotransmitter at muscarinic receptors is acetylcholine, which in vertebrates functions at cholinergic synapses in both the peripheral and central nervous system (Brown and Taylor (1996) in Goodman and Gilman's The Pharmacological Basis of Therapeutics, eds.

- 5 Hardman, J.G. & Limbird, L.E., McGraw Hill, pp. 141-160). Acetylcholine has a wide variety of functions in vertebrate signaling including sympathetic and parasympathetic ganglion cells as well as the adrenal medulla, synapses within the central nervous system, and motor end plates on skeletal muscle innervated by somatic motoneurons (Brown and Taylor (1996) in Goodman and Gilman's The
- 10 Pharmacological Basis of Therapeutics, eds. Hardman, J.G. & Limbird, L.E., McGraw Hill, pp. 141-160). Muscarinic receptors are found in muscle, the autonomic ganglia, the central nervous system and secretory glands. These receptors couple to G proteins and signal on longer time scales than nicotinic receptors. Signaling can be either excitatory or inhibitory (Lefkowitz et al. (1996)
- 15 in Goodman and Gilman's The Pharmacological Basis of Therapeutics, eds. Hardman, J.G. & Limbird, L.E., McGraw Hill, pp. 105-139). Both muscarinic and nicotinic receptors have been found in invertebrates such as *Drosophila* and *C. elegans* as well as vertebrates (Lewis et al. (1980) Genetics 95:905-928; Dudai and Ben-Barak (1977) FEBS Lett. 81:134-136; Haim et al. (1979) J. Neurochem.
- 20 32:543-522; and Culotti and Klein (1983) J. Neurosci. 3:359-368).

The nicotinic receptor has been the primary focus of the studies on cholinergic signaling in the worm. The drug levamisole, a nicotinic agonist, is toxic to animals, causing muscle hypercontraction (Lewis et al. (1980) Genetics 95:905-928; and Lewis et al. (1980) Neuroscience 5:967-989). Mutants that are

25 resistant to this drug have revealed components of a nicotinic signaling cascade

(Lewis et al. (1980) Genetics 95:905-928; and Lewis et al. (1980) Neuroscience 5:967-989). Levamisole has no effect on dauer recovery, suggesting that the nicotinic receptor pathway does not regulate dauer arrest.

Fewer studies, however, have been done on muscarinic signaling in *C. elegans*. Binding studies on crude homogenates of *C. elegans* have shown that they contain muscarinic receptors that have the potential to bind to the muscarinic ligands, [3H] QNB (Yamamura & Snyder (1974) Proc. Natl. Acad. Sci. 2 :1725-1729) and [3H] N-methylscopolamine (Burgermeister et al. (1978) Mol. Pharmacol. 14:240-256) with high affinity (Culotti and Klein (1983) J. Neurosci. 3:359-368). These receptors were found in both *C. elegans* adults and L1 and L2 larvae ((Culotti and Klein (1983) J. Neurosci. 3:359-368). Several potential muscarinic receptor homologues have been identified in the *C. elegans* genome sequence database (Sulston et al. (1992) Nature 356:37-41)

There are two different classes of muscarinic receptor agonists: choline esters and cholinomimetic alkaloids. Both arecoline and pilocarpine are naturally occurring drugs from the betel nut seed and the Pilocarpus leaf, respectively, while oxotremorine is a synthetic drug (Brown and Taylor (1996) in Goodman and Gilman's The Pharmacological Basis of Therapeutics, eds. Hardman, J.G. & Limbird, L.E., McGraw Hill, pp. 141-160). Carbachol is a synthetic choline ester which mimics acetylcholine and acts at both muscarinic and nicotinic receptors in mammals (Brown and Taylor (1996) in Goodman and Gilman's The Pharmacological Basis of Therapeutics, eds. Hardman, J.G. & Limbird, L.E., McGraw Hill, pp. 141-160). Arecoline, pilocarpine, and oxotremorine are drugs that have the same sites of action and function as the choline esters (Brown and Taylor (1996) in Goodman and Gilman's The Pharmacological Basis of

Therapeutics, eds. Hardman, J.G. & Limbird, L.E., McGraw Hill, pp. 141-160).

Arecoline also acts on nicotinic receptors. Atropine specifically inhibits mammalian muscarinic responses (Brown and Taylor (1996) in Goodman and Gilman's The Pharmacological Basis of Therapeutics, eds. Hardman, J.G. & Limbird, L.E., McGraw Hill, pp. 141-160). Since all of the drug-induced dauer recovery was inhibited by atropine, we concluded that this response was mediated by muscarinic signaling.

Molecular analysis of the dauer mutants revealed that a TGF- $\beta$  signaling pathway regulated dauer arrest (Figure 46). Mutations in *daf-7*, which encodes a TGF- $\beta$  ligand, caused animals to arrest at the dauer stage even under favorable growth conditions (Ren et al. (1996) Science 274:1389-1391). The same phenotype was observed in animals bearing a mutation in either of the two TGF- $\beta$  receptors, *daf-1* and *daf-4* (Georgi et al. (1990) Cell 61:635-645; and Estevez et al. (1993) Nature 365:644-649; Figure 46). Downstream of the receptors are members of the Smad signaling group including the genes *daf-8*, *daf-14* and *daf-3* (Figure 46). Muscarinic agonists potently induced recovery of dauer larvae induced by mutations in this group of genes (Figure 44A-44C).

An insulin-like signaling pathway represented by *daf-2* and *age-1* functions in parallel to this TGF- $\beta$  pathway (Riddle et al. (1981) Nature 290:668-671; Vowels and Thomas (1992) Genetics 130:105-123; Thomas et al. (1993) Genetics 134:1105-1117; Gottlieb and Ruvkun (1994) Genetics 137:107120; Kimura et al. (1997) Science 277:942-946; and Morris et al. (1996) Nature 382:536-539.41; Figure 46). *daf-2* is a member of the insulin receptor family (Kimura et al. (1997) Science 277:942-946) and *age-1* encodes phosphatidylinositol (PI)-3- kinase (Morris et al. (1996) Nature 382:536-539.41)

suggesting that the level of an insulin-like molecule is down-regulated during pheromone-induced dauer arrest. None of the drugs tested, including the muscarinic agonists and antagonists, could induce dauer recovery in *daf-2* mutants (Figure 44A-44C). Thus the cholinergic input to dauer recovery depends on insulin-like signaling. We suggest that muscarinic agonists induce recovery of the TGF- $\beta$  pathway mutant dauer larvae or pheromone-induced dauer larvae by stimulating signaling in the *daf-2* insulin-like pathway. In this way, cholinergic stimulation can induce recovery in animals with defective TGF- $\beta$  pathway genes but not in animals with defect insulin-like pathway genes.

In vertebrate insulin signaling, many studies link muscarinic and insulin signaling pathways. Both adrenergic and cholinergic fibers innervate secretory cells in the vertebrate islet of Langerhans (Ahren et al. (1986) *Diabetologia* 29:827-836; and Yamamura and Snyder (1974) *Proc. Natl. Acad. Sci.* 71:1725-1729). Consistent with the suggestion that muscarinic inputs increase *C. elegans* insulin-like signaling, mammalian autonomic cholinergic fibers enhance insulin secretion. Pharmacological stimulation with acetylcholine or carbachol can induce insulin release both *in vivo* and *in vitro*. This induction is completely abolished by atropine, showing that it is mediated by activation of muscarinic receptors on the  $\beta$  cells (Ahren et al. (1986) *Diabetologia* 29:827-836; Boschero et al. (1995) *Am. J. Physiol.* 268:E336-E342; and Latifpour et al. (1992) *J. Urol.* 147:760-763). In mammalian systems, binding of acetylcholine to the  $\beta$  cell muscarinic receptor causes activation of sodium channels, which in turn leads to a change in membrane potential to induce insulin.

These data suggest the model shown in Figure 46 for dauer recovery in *C. elegans*. When pheromone levels decrease and food levels increase,

acetylcholine is secreted from an as yet unidentified neuron and binds to the muscarinic receptor on an insulin-like secreting neuron or other cell. This induces secretion of an insulin-like signal to in turn induce dauer recovery (Figure 46). The lack of muscarinic induced dauer recovery in *daf-2* mutants suggest that the insulin-like dauer recovery signal acts via the DAF-2 receptor homologue. From analogy with the vertebrate studies, we suggest that a muscarinic signal causes an increase in insulin release that would bind to the DAF-2 receptor and activate downstream genes which promote dauer recovery. We suggest that the insulin-like DAF-2 ligand is produced by neurons just as the DAF-7 TGF- $\beta$  signal is produced by the ASI sensory/secretory neuron. Insulin secreting pancreatic  $\beta$ -cells have many neuronal features and are thought to be specialized "ganglia" related to the enteric nervous system of lower vertebrates. In addition, proteins related to insulin are produced by metabolism regulating neurons in *Limulus*. Distant relatives of insulin are found in the *C. elegans* genome database. We suggest that the secretory cells that express such an insulin-like gene will also express muscarinic receptors and be connected to food, pheromone, and temperature sensory neurons.

Temperature acts as a modulator for dauer recovery (Riddle and Albert (1997) in *C. elegans* II, eds. Riddle, D.L., Blumenthal, T., Meyer, B.J. & Priess, J.R., Cold Spring Harbor Laboratory Press, pp. 739-768, Figure 45A-B). The thermoregulatory circuit for temperature sensation and output of that information to motor and endocrine pathways has been identified (Hobert et al. (1997) *Neuron* 19:345-357). This pathway consists of the thermosensory neuron AFD coupled to the interneurons AIY and AIZ (Hobert et al. (1997) *Neuron* 19:345-357; and Henquin (1994) in *Joslin's Diabetes Mellitus*, eds. Kahn, C.R. & Weir, G.C., (Lea

& Febiger, pp. 56-80)). Mutations in the gene *ttx-3*, which affect AIY function and is expressed exclusively in the AIY interneurons (Hobert et al. (1997) Neuron 19:345-357), decouple this thermoregulatory pathway from the dauer pathway: *daf-7; ttx-3* double mutant animals form dauers that recover at high temperature, unlike *daf-7* single mutants (Hobert et al. (1997) Neuron 19:345-357). However, *daf-2; ttx-3* double mutant dauers do not recover at high temperature, like the *daf-2* mutant alone. We suggest that thermosensory signals through the thermoregulatory AIY and AIZ interneurons couple via as yet unidentified insulin-like secretory neurons (Figure 46). Given that rates of growth and metabolism are intimately connected to cultivation temperature in invertebrates, the coupling of thermosensation to metabolic control is reasonable. Such a coupling of thermosensory input to metabolic control by the *daf-2* insulin-like signaling pathway is analogous to the hypothalamic modulation of autonomic input to the pancreatic beta cells.

The muscarinic signaling pathway also acts in recovery of hookworm infective L3 from their arrested "dauer" state. Recovery from dauer arrest in hookworm occurs in the definitive host in response to an undefined host-specific signal. We suggest that upregulation of an insulin-like molecule by a cholinergic pathway also causes dauer recovery upon entry into the host in *A. caninum*.

Accordingly, such parasite insulin-like signals provide targets for anti-helminthic drugs. For example, known muscarinic signaling drugs may constitute novel chemotherapeutic strategies to perturb the dauer maintenance process in invertebrate hosts as well as the recovery process in human hosts.

### Other Screening Assays

Other drug screening assays may also be performed using either *C. elegans* worms or mammalian cell cultures. If desired, such assays may include the use of reporter gene constructs.

For example, evaluation of the effects of test compounds on dauer formation or reporter gene expression in mutant *C. elegans* strains expressing particular human homologs of the *daf*, *age*, or *akt* genes (i.e., humanized *C. elegans*) represent useful screening methods. Expression of the human homologs in *C. elegans* is accomplished according to standard methods and, if desired, such genes may be operatively linked to a gene promoter obtained from *C. elegans*.

Such promoters include, without limitation, the *C. elegans daf-16*, *age-1*, *daf-3*, *daf-4*, and *akt* gene promoters. For example, the 2.5 kb *age-1* promoter can be generated and isolated by employing standard PCR methods using the following primers: 5'GGAAATATTTTAGGCCAGATGCG3' (SEQ IS NO: 49) and 5'CGGACAGTCCTGAATACACC3' (SEQ ID NO: 50).

Additionally, mammalian tissue culture cells expressing *C. elegans daf*, *age-1*, or *akt* homologs may be used to evaluate the ability of a test compound or extract to modulate the insulin or TGF- $\beta$  signaling pathways. Because the signaling pathways from the ligands, receptors, kinase cascades, and downstream transcription factors are conserved from man to worm, test compounds or extracts that inhibit or activate the worm signaling proteins should also inhibit or activate their respective human homolog. For example, our identification that DAF-16 is a transcription factor that acts downstream of insulin-like signaling in *C. elegans* indicates that human DAF-16 transcription reporter genes also can be used to identify drugs that inhibit any of the kinases in the signaling pathway downstream of insulin signaling. For example, the use of DAF-16 and DAF-3 protein binding

sites in reporter gene constructs may be used to monitor insulin signaling.

Candidate compounds mimicking insulin signaling (e.g., PIP3 agonists) are expected to increase reporter gene expression and are considered useful in the invention.

## 5 **Reporter Gene Construct**

In one particular example, the invention involves the use of a reporter gene that is expressed under the control of a *C. elegans* gene promoter, e.g., a promoter that includes the TCTCGTTGTTTGCCGTCGGATGTCTGCC (SEQ ID NO: 51) enhancer element, such as the *C. elegans* pharyngeal myosin promoter  
10 (Okkema and Fire, *Development* 120: 2175-2186, 1994). This enhancer element is known to respond to DAF-3 regulation (i.e., in *daf-7* mutants, where *daf-3* is active, the element confers low level expression to reporter genes; whereas in a *daf-7*; *daf-3* mutant (for example, *daf-7* (e1372); *daf-3*), the element confers low level expression to reporter genes). Other equivalent enhancer elements may also  
15 be used in the invention, e.g., the enhancer element which is bound by the *Xenopus* Smad1 and Fast1 forkhead proteins (*Nature* 383 600-608, 1996). The enhancer element is cloned upstream of any standard reporter gene, e.g., the luciferase or green fluorescent protein (GFP) reporter genes. In preferred embodiments, the GFP reporter gene is used in *C. elegans*. In other preferred embodiments, either  
20 the GFP or the luciferase reporter genes may be used in a mammalian cell based assay. The reporter gene construct is subsequently introduced into an appropriate host (e.g., *C. elegans* or a mammalian cell) according to any standard method known in the art. Analysis of reporter gene activity in the host organism or cell is determined according to any standard method, e.g., those methods described

herein. Such reporter gene (and host cell systems) are useful for screening for drugs that modulate insulin or DAF-7 metabolic control signaling.

In addition, any number of other transcriptional fusions to fat based metabolic genes, such as fatty acid synthase, hormone sensitive lipase orthologue, and acetyl coa synthase, may also be constructed. These genes are expected to be up regulated when the animals shift to fat based metabolism and to be directly regulated by DAF-16 and DAF-3, and perhaps DAF-12.

Transcriptional fusions to GFP allow the screening of drugs or mutants for altered regulation of these genes and altered metabolism. Such drugs or gene targets are useful in the control of obesity and diabetes. For example, drugs that inactivate the expression of fat synthesis genes in *C. elegans* may be used to treat diabetes and obesity. Similarly, full length protein fusions of these genes to GFP reveal the subcellular localization of the proteins. Drugs or mutants that perturb the cell biology of these proteins also provide useful treatments and drug targets for obesity control as well as diabetes.

Shown below are conserved protein regions of *C. elegans* homologues of key metabolic enzymes (SEQ ID NOS: 211-363). GFP fusions may be constructed using the 5' promoter regions located between these conserved protein domains and the next gene located 5' to these regions, as described above for the glucose transporter GFP fusion gene.

### Pepck

>R11A5

Length = 26,671

Plus Strand HSPs:

Score = 994 (461.5 bits), Expect = 0.0, Sum P(5) = 0.0  
Identities = 176/223 (78%), Positives = 195/223 (87%), Frame = +1

Query: 201 AKNNGEFVRCVHVSQGPKPVATKVINHWPCNPEKTIIAHRPAEREIWSFGSGYGGNSLLG 260  
A N +FVRC+HVSQ P+PV +VINHWPCNPE+ +IAHRP EREIWSFGSGYGGNSLLG  
5 Sbjct: 8682 ALGNQDFVRCIHSVGLPRPVKQRVINHWPCNPERVLIHRPPEREIWSFGSGYGGNSLLG 18861

Query: 261 KKCFFALRIAMNIGYDEGWMAEHMLIMGVTSKGEERFVAAAFPSACGKTNLAMLEPTIPG 320  
KKCFALRIA NI DEGWMMAEHMLIMGVT P G E F+AAAFPSACGKTNLAMLEPT+PG  
Sbjct: 18862 KKCFFALRIASNIAKDEGWMAEHMLIMGVTRPCGREHFIAAFPSACGKTNLAMLEPTLPG 19041  
Query: 321 WKVRVIGDDIAWMKFGADGRLYAINPEYGGFFGVAPGTSKTNPMAMASFQENTIFTNVAE 380  
10 WKVR +GDDIAWMKFG DGRLYAINPE GFFGVAPGTS+KTNPMA+A+FQ+N+IFTNVAE  
Sbjct: 19042 WKVRCVGDDIAWMKFGEDGRLYAINPEAGFFGVAPGTSNKTNPMAVATFQKNSIFTNVAE 19221

Query: 381 TADGEYFWEGLEHEVKNPKVDMINWLGEPPWHIGDESKAAHPNS 423  
TA+GEYFWEGLE E+ + VD+ WLGE WHIG+ AAHPNS  
Sbjct: 19222 TANGEYFWEGLEDEIADKNVDITTLWGEKWHIGEPGVAAHPNS 19350

15 Score = 657 (305.1 bits), Expect = 0.0, Sum P(5) = 0.0  
Identities = 120/173 (69%), Positives = 144/173 (83%), Frame = +1

Query: 32 KGDFVSLPKHVQRFVAKAEELMKPSAIFICDGSQNEADELIARCVERGVLPKAYKNKY 91  
+GDF LP VQRF+AEKAEELM+P IFICDGSQ+EADELI + +ERG+L L+AY+NNY  
Sbjct: 18181 QGDFHLLPAKVQRFIAEKAEELMRPRGIFICDGSQHEADELIDKLIERGMLSKLEAYENNY 18360

20 Query: 92 LCRTDPRDVARVESKTMITPEKYDSVCHTPEGVKPMMQWMSPEFGKELDDRFPFGCMA 151  
+CRTDP+DVARVESKTM+T KYD+V HT EGV+P+MG W++P++ ELD RFPFGCMA  
Sbjct: 18361 ICRTDPKDVARVESKTMVTKNKYDTVTHTKEGVEPIMGHWLAPEDLATELDSRFPFGCMA 18540

Query: 152 GRTMYVIPYSMGPVGGPLSKIGIELTDSYVVLCMRIMTRMGEPVLKALAKNN 204  
GR MYVIP+SMGPVGGPLSKIGI+LTDS+YVVL MRIMTR+ V AL +  
25 Sbjct: 18541 GRIMYVIPFSMGPVGGPLSKIGIQLTDSNYVVLMSRIMTRVNNDVWDALGNQD 18699

Score = 453 (210.3 bits), Expect = 0.0, Sum P(5) = 0.0  
Identities = 77/107 (71%), Positives = 90/107 (84%), Frame = +1

Query: 424 RFTAPAGQCPIIHPDWEKPEGVPIDAIIFGGRRPEGVPLVFESRSWVHGIFVGACVKSEA 483  
 RF APA QCPIIHPDWE P+GVPI+AIIFGGRRP+GVPL++E+ SW HG+F G+C+KSEA  
 Sbjct: 19396 RFAAPANQCPIIHPDWESPQGVPIEAIIFGGRRPQGVPLIYETNSWEHGVFTGSCLKSEA 19575

5 Query: 484 TAAAEHTGKQVMHDPAMRPFMGYNFGRYMRHWMKLGQPPHKVPKIF 530  
 TAAAE TGK VMHDPAMRPFMGYNFG+Y++HW+ L KV F  
 Sbjct: 19576 TAAAEFTGKTVMHDPAMRPFMGYNFGKYLQHWLDLKTDSRKVIDFF 19716

Score = 404 (187.6 bits), Expect = 0.0, Sum P(5) = 0.0  
 Identities = 68/116 (58%), Positives = 89/116 (76%), Frame = +1

10 Query: 526 VPKIFHVNWFRQSADHKFLWPGYGDNIRVIDWILRRCSGDATIAEETPIGFIPKKGTINL 585  
 +PKI+HVNWFR+ +++KFLWPG+GDNIRVIDWI+RR G+ I ETPIG +P KG+INL  
 Sbjct: 19750 MPKIYHVNWFRKDSNNKFLWPGFGDNIRVIDWIIRRLDGEQEIGVETPIGTVPKGSINL 19929

Query: 586 EGLPNVNWDELMSIPKSYWLEDVETKTFENQVGSDDLPEIAKELEAQTERIKAL 641  
 EGL VNWDELMS+P YW +D E + F + QVG DLP + E++AQ +R++ L  
 Sbjct: 19930 EGLGEVNWDELMSVPADYWKQDAQEIRKFLDEQVGEDLPEPVRAEMDAQEKRVQTL 20097

15 Score = 69 (32.0 bits), Expect = 0.0, Sum P(5) = 0.0  
 Identities = 15/36 (41%), Positives = 21/36 (58%), Frame = +1

Query: 5 SLSHFKDDDFAVVSEVVTHKQNHIPVIKGDVSLPK 40  
 SL +D F VV+EVV + H+P++K F S K  
 Sbjct: 14722 SLRQISEDAFYVNEVVMKRLGHVPILKVIFESSEK 14829

20 Score = 39 (18.1 bits), Expect = 6.9e-244, Sum P(4) = 6.9e-244  
 Identities = 9/25 (36%), Positives = 11/25 (44%), Frame = +3

Query: 148 GCMAGRTMYVIPYSMGPVGGPLSKI 172  
 GC R + V P S PL K+  
 Sbjct: 8040 GCSGRRVLCVPCSHSSSALPLQKV 8114

25 Score = 38 (17.6 bits), Expect = 4.0e-285, Sum P(5) = 4.0e-285  
 Identities = 7/16 (43%), Positives = 9/16 (56%), Frame = +1

Query: 588 LPNVNWDELMSIPKSY 603  
L + NW +S P SY  
Sbjct: 22654 LESFNWFSFVSCPDSY 22701

Score = 37 (17.2 bits), Expect = 2.0e-48, Sum P(3) = 2.0e-48  
5 Identities = 6/14 (42%), Positives = 9/14 (64%), Frame = +1

Query: 117 SVCHTPEGVKPMMG 130  
+V H P ++P MG  
Sbjct: 19603 TVMHDPAMRPFMG 19644

### Acetyl coa carboxylase

10 >W09B6  
Length = 32,900  
Plus Strand HSPs:  
  
Score = 562 (259.1 bits), Expect = 0.0, Sum P(14) = 0.0  
Identities = 109/197 (55%), Positives = 138/197 (70%), Frame = +2

15 Query: 1951 SGFFDYGSFSEIMQPWAQTVVVGRRARLGGIPVGVVAVETRTVELSVPADPANLDSEAKII 2010  
+G D SF EI WA+++V GRARL GIP+GVV+ E R VPADPA S+ +  
Sbjct: 28280 TGICDTMSFDEICGDWAKSIVAGRARLCGIPIGVVSSEFRNFSTIVPADPAIDGSQVQNT 28459

Query: 2011 QQAGQVWFPSAFKTYQAIKDFNREGLPLMVFANWRGFSGGMKMYDQVLKFGAYIVDGL 2070  
Q+AGQVW+PDSAFKT +AI D N+E LPLM+ A+ RGFSGG KMYD VLKFGA IVD L

20 Sbjct: 28460 QRAGQVWYPDSAFKTAEAINDLNKENLPLMIIASLRGFSGGQKMYDMVLKFGAQIVDAL 28639

Query: 2071 RECSQPVVMVYIPQAE LRGGSWVVIDPTINPRHMEMYADRESRGSVLEPEGTVEIKFRKK 2130  
++PV+VYIP ELRGG+W V+D I P + + AD +SRG +LEP V IKFRK  
Sbjct: 28640 AVYNRPVIVYIPEAGELRGGAWAVLDSKIRPEFIHLVADEKSRGGILEPNAVVGIFRKP 28819

Query: 2131 DLVKTMRRVDPVYIRLA 2147  
+++ M+R DP Y +L+

25 Sbjct: 28820 MMEMMKRSDPTYSKLS 28870

Score = 357 (164.6 bits), Expect = 0.0, Sum P(14) = 0.0  
Identities = 68/124 (54%), Positives = 89/124 (71%), Frame = +2

5 Query: 303 VGYPVMIKASEGGGKGIRKVNADDFPNLFRQVQAEVPGSPIFVMRLAKQSRHLEVQIL 362  
+G+P+MIKASEGGGKGIRK +DF ++F +V EV GSPIF+M+ +RH+EVQ+L  
Sbjct: 23264 IGFLPMIKASEGGGKGIRKCTKVEDFKSMFEEVAQEVQGSPIFLMKCVGDGARHIEVQLL 23443

Query: 363 ADQYGNAILSLFGRDCSVQRRHQXXXXXXXXXXXXXVFEHMEQCAVKLAKMVGYSAGTV 422  
AD+Y N IS++ RDCS+QRR QK + + M++ AV+LAK VGY SAGTV  
Sbjct: 23444 ADRYENVISVYTRDCSIQRRCKIIEEAPAIASSHIRKSMQEDAVRLAKYVGYESAGTV 23623

10 Query: 423 EYLY 426  
EYLY  
Sbjct: 23624 EYLY 23635

Score = 345 (159.1 bits), Expect = 0.0, Sum P(14) = 0.0  
Identities = 65/116 (56%), Positives = 86/116 (74%), Frame = +2

15 Query: 1787 KEEGLGAENLRGSGMIAGESSLAYDEIITISLVTCAIGIGAYLVRGQRTIQVENSHLI 1846  
K E +G ENL+GSG+IAGE++ AY E+ T VT R++GIGAY RL R +Q + SHLI  
Sbjct: 27794 KNEKIGVENLQSGSLIAGETARAYA EVPTYCYVTGRSVGIGAYTARLAHRIVQHKQSHLI 27973

Query: 1847 LTGAGALNKVLGREVYTSNNQLGGIQIMHNNGVTHCTVCDDFEGVFTVLHWLSYMP 1902  
LTG ALN +LG++VYTSNNQLGG ++M NGVTH V +D EG+ V+ W+S++P  
Sbjct: 27974 LTGYEALNTLLGKKVYTSNNQLGGPEVMFRNGVTHAVVDNDLEGIKAVIRWMSFLP 28141

20 Score = 319 (147.1 bits), Expect = 0.0, Sum P(14) = 0.0  
Identities = 59/119 (49%), Positives = 80/119 (67%), Frame = +2

Query: 503 HVIAARITSENPDEGFKPSSGTVQELNFRSNKNVWGYFSVAAAGGLHEFADSQFGHCFSW 562  
H IAARIT ENPD+ F+PS+G V E+NF S+++ W YFSV +H+FADSQFGH F+  
Sbjct: 23870 HAIAARITCENPDDSRPSTGKVYEINFSSQDAWAYFSVGRGSSVHQFADSQFGHIFTR 24049

25 Query: 563 GENREEAISNMVVALKELSIRGDFRTTVEYLIKLETESFQLNRIDTGWLDRLIAEKVQ 621  
G +R EA++ M LK ++IR F T V YL+ L+ F N +T WLD+ IA K++  
Sbjct: 24050 GTSRTEAMNTMCSTLKHMTIRSSFPTQVNYLVLDLMHDADFINNAFNTQWLDKRIAMKIK 24226

Score = 303 (139.7 bits), Expect = 0.0, Sum P(14) = 0.0  
Identities = 55/90 (61%), Positives = 70/90 (77%), Frame = +2

Query: 178 PGGANNNNYANVELILDIAKRIPVQAVWAGWGHASENPKLPELLLKNGIAFMGPPSQAMW 237  
P G N NN+ANV+ IL A + V AVWAGWGHASENP LP L + IAF+GPP+ AM+  
5 Sbjct: 22886 PSGTNKNNFANVDEILKHAIKYEVDVWAGWGHASENPDLPRRLNDHNIAFIGPPASAMF 23065

Query: 238 ALGDKIASSIVAQTAGIPTLPWSGSGLRVD 267  
+LGDKIAS+I+AQT G+PT+ WSGSG+ ++  
Sbjct: 23066 SLGDKIASTIIAQTVGVPTVAWSGSGITME 23155

### Trehelase

10 >C23H3

Length = 39,721  
Minus Strand HSPs:

Score = 227 (104.5 bits), Expect = 1.0e-95, Sum P(6) = 1.0e-95  
Identities = 36/67 (53%), Positives = 51/67 (76%), Frame = -2

15 Query: 2 VIKNLGYMVDNHGFVPNGGRVYYLTRSQPPLLTPMVYEYYMSTGDLDFVMEILPTLDKEY 61  
+I N +++++ GFVPNGGRVYYL RSQPP PMVYEYY++T D+ V +++P ++KEY  
Sbjct: 9798 MILNFAHIIETYGFVPNGGRVYYLRRSQPPFFAPMVYEYYLATQDIQLVADLIPVIEKEY 9619

Query: 62 EFWIKNR 68  
FW + R

20 Sbjct: 9618 TFWSEERR 9598

Score = 182 (83.8 bits), Expect = 1.0e-95, Sum P(6) = 1.0e-95  
Identities = 32/92 (34%), Positives = 55/92 (59%), Frame = -2

Query: 146 MDSIRTWSIIPADLNAFMCANARILASLYEIAAGDFKKVKVFEQRYTWAKREMRELHWNEN 205  
+ +I T +I+P DLNAF+C N I+ Y++ G+ K + R+T + ++ +  
25 Sbjct: 9372 ISTIETTNIVPVDLNAFLCYNMNIMQLFYKLTGNPLKHLEWSSRFTNFREAF TKVFYVPA 9193

Query: 206 DGIWYDYDIELKTHSNQYYVSNAPPLYAKCYD 237  
WYDY++ TH+ ++ SNAVPL+++CYD

Sbjct: 9192 RKGWYDYNLRTLTHNTDFFASNAVPLFSQCYD 9097

Score = 178 (81.9 bits), Expect = 1.0e-95, Sum P(6) = 1.0e-95  
Identities = 37/102 (36%), Positives = 55/102 (53%), Frame = -2

Query: 246 VHDYLERQGLLKYTEGLPTSLAMSSTQQWDKENAWPPMIHVMIEGFRRTTGDIKLMKVAEK 305

5

V++ ++ G G+PTS+ + QQWD N W PM HM+IEG R + + L + A

Sbjct: 9069 VYNEMQNSGAFSIPGGIPTSMNEETNQQWDFPNGWSPMNHMIEGLRKSNNPILQQKAFT 8890

Query: 306 MATSWLTGTYQSFIETHAMFEKYNVTPHTEETSGGGGGGEYEV 347

+A WL Q+F + M+EKYNV + + GG E +V

Sbjct: 8889 LAEKWLETNMQTFNVSDMWEKYNVKEPLGKLATGGEYEVQV 8764

10

Score = 169 (77.8 bits), Expect = 1.0e-95, Sum P(6) = 1.0e-95  
Identities = 29/58 (50%), Positives = 41/58 (70%), Frame = -2

Query: 84 YQYKAKLKVPRPESYREDSELAEHLQTEAEKIQMWSEIASAAETGWDFSTRWFSONGD 141

+QY+ + + PRPES+RED AEH T+ K Q + ++ SAAE+GWDFS+RWF + D

Sbjct: 9546 FQYRTEAETPRPESFREDVLSAEHFTTKDRKKQFFKDLGSAAESGWDFSSRWFKNHKD 9373

15

Score = 76 (35.0 bits), Expect = 1.0e-95, Sum P(6) = 1.0e-95  
Identities = 13/21 (61%), Positives = 15/21 (71%), Frame = -1

Query: 348 QTGFGWTNGVILDLLDKYGDQ 368

Q GFGWTNG LDL+ Y D+

Sbjct: 8722 QAGFGWTNGAALDLIFTYSR 8660

20

Score = 45 (20.7 bits), Expect = 1.0e-95, Sum P(6) = 1.0e-95  
Identities = 10/24 (41%), Positives = 15/24 (62%), Frame = -1

Query: 371 SSSTASKFSFSLSNITFVVFI LYI 394

+SS++S F +S VF+LYI

Sbjct: 8545 TSSSSSTFGYSNITLITFVFLYI 8474

25

Score = 38 (17.5 bits), Expect = 2.6e-98, Sum P(7) = 2.6e-98  
Identities = 7/7 (100%), Positives = 7/7 (100%), Frame = -2

Query: 342 GGEYEVQ 348

GGEYEVQ

Sbjct: 8787 GGEYEVQ 8767

Score = 37 (17.0 bits), Expect = 1.6e-19, Sum P(4) = 1.6e-19

5 Identities = 8/18 (44%), Positives = 10/18 (55%), Frame = -2

Query: 217 KTHSNQYYVSNAPLYAK 234

K ++ YYVS P Y K

Sbjct: 30345 KFTAHPIYVSRTPPRYHK 30292

>W05E10

10 Length = 31,273

Minus Strand HSPs:

Score = 224 (103.1 bits), Expect = 7.0e-90, Sum P(7) = 7.0e-90

Identities = 43/67 (64%), Positives = 49/67 (73%), Frame = -1

Query: 2 VIKNLGYMVDNHFVPNGGRVYYLTRSQPPLTPMVYEYIMSTGDLDFVMEILPTLDKEY 61

15 +I+NL MVD +GFVPNGGRVYYL RSQPP L MVYE Y+ T D FV E+LPTL KE

Sbjct: 28957MIRNLASMDKYGFVPNGGRVYYLQRSQPPFLAAMVYELYEATNDKAFVAELLPTLLKEL28778

Query: 62 EFWIKNR 68

FW + R

Sbjct: 28777 NFWNEKR 28757

20 Score = 192 (88.4 bits), Expect = 7.0e-90, Sum P(7) = 7.0e-90

Identities = 31/84 (36%), Positives = 52/84 (61%), Frame = -3

Query: 154 IIPADLNAFMCANARILASLYEIAGDFKKVKVFEQRYTWAKREMRELHWNEDGIWYDYD 213

++P DLN + C N I + LYE GD K ++F + + ++ + +N TDG WYDY+

Sbjct: 2842 7VLPVDLNGLLCWNMDIMEYLYEQIGDTKNSQIFRNKRADFRDTVQNVFYNRDGTWYDYN 28248

25 Query: 214 IELKTHSNQYYVSNAPLYAKCYD 237

+ ++H+ ++Y S AVPL+ CY+

Sbjct: 28247 LRTQSHNPRFYTSTAVPLFTNCYN 28176

Score = 125 (57.5 bits), Expect = 7.0e-90, Sum P(7) = 7.0e-90  
Identities = 20/48 (41%), Positives = 30/48 (62%), Frame = -2

Query: 249 YLERQGLLK YTKGLPTSLAMSSTQQWDKENAWPPMIH MVIEGFRTTGD 296  
+ ++ G+ Y G+PTS++ S QQWD N W P HM+IEG R + +

5 Sbjct: 28092 FFQKMGVFTYPGGIPTSMSQESDQQWDFPNGWSPNNHMIIEGLRKSAN 27949

Score = 90 (41.4 bits), Expect = 7.0e-90, Sum P(7) = 7.0e-90  
Identities = 15/18 (83%), Positives = 18/18 (100%), Frame = -2

Query: 120 EIASAAETGWDFSTRWFS 137  
++ASAAE+GWDFSTRWFS

10 Sbjct: 28566 DLASAAESGWDFSTRWFS 28513

Score = 89 (41.0 bits), Expect = 7.0e-90, Sum P(7) = 7.0e-90  
Identities = 18/40 (45%), Positives = 24/40 (60%), Frame = -1

Query: 79 KQFPYYQYKAKLKVPRPESYREDS ELAEHLQTEAEKI QMW 118  
K F YQYK VPRPESYR D++ + L A++ Q +

15 Sbjct: 28732 KSFKVYQYKTASN VPRPESYRVDTQNSAKLANGADQQQFY 28613

Score = 77 (35.4 bits), Expect = 7.0e-90, Sum P(7) = 7.0e-90  
Identities = 14/21 (66%), Positives = 16/21 (76%), Frame = -3

Query: 348 QTGFGWTNGVILDLLDKYGDQ 368  
Q GFGW+NG ILDLL Y D+

20 Sbjct: 24395 QDGFGWNSGAILDLLLTYNDR 24333

Score = 51 (23.5 bits), Expect = 7.0e-90, Sum P(7) = 7.0e-90  
Identities = 11/27 (40%), Positives = 16/27 (59%), Frame = -3

Query: 365 YGDQFASSSTASKFSFSLSNITFV VFI 391  
Y FASSS AS FS +++ F + +

25 Sbjct: 2846 YN\*PFASSSDASSCPFSTNSVIFSILV 2766

Score = 41 (18.9 bits), Expect = 3.3e-93, Sum P(8) = 3.3e-93

Identities = 7/9 (77%), Positives = 8/9 (88%), Frame = -2

Query: 340 GGGGEYEVQ 348

G GGEY+VQ

Sbjct: 24468 GSGGEYDVQ 24442

5 Score = 39 (18.0 bits), Expect = 2.0e-37, Sum P(5) = 2.0e-37  
Identities = 7/14 (50%), Positives = 8/14 (57%), Frame = -2

Query: 221 NQYYVSNAPLYAK 234

N YY+ V LY K

Sbjct: 4524 NHYYIIQMVSPLYTK 4483

10 Score = 38 (17.5 bits), Expect = 4.0e-88, Sum P(7) = 4.0e-88  
Identities = 11/30 (36%), Positives = 13/30 (43%), Frame = -1

Query: 367 DQFASSSTASKFSFSLSNITFVVFILYIFS 396

DQF S SKFS + F +FS

Sbjct: 7588 DQFVISFICKSFSSKNKKLYFCPSHFLFS 7499

15 Gene fusions to GFP may also be constructed using, for example, the  
isocitrate dehydrogenase and isocitrate lyase genes to test for transition to the  
glyoxylate cycle for the generation of glucose from fatty acid metabolism during  
dauer arrest and recovery. Moreover, gene fusions to hexokinase and glucose  
metabolism genes may be used test for the switch to sugar based metabolism  
20 during reproductive development.

GFP fusions to these genes are expected to be transcriptionally regulated  
depending on whether the animal is in fat storage, fat breakdown, glycogen  
storage, or glycogen breakdown, trehalose storage, or trehalose breakdown  
metabolic states. Drugs that perturb the expression of these genes may regulate  
25 transcriptional regulatory proteins like DAF-3, DAF-12, and DAF-16 that may

regulate batteries of such metabolic genes. The GFP reporter genes provide a screen for perturbations of these regulatory genes. In addition, GFP fusions to the full length proteins may also reveal subcellular localization, for example, of fat storage proteins to fat droplets and regulation of the localization of these proteins.

- 5 Drugs that perturb the localization of these fusion proteins may also be potent regulators of fat metabolism and may be used to treat obesity and diabetes.

### **Daf12-GFP Fusions**

*Daf-12* expression has been examined using a full length, rescuing GFP fusion to *daf-12*. We have found that the gene is expressed in a small number of  
10 neurons in wild type animals, and many more in a *daf-2*, *daf-7*, or pheromone induced dauer. Thus, the *daf-12* expression pattern is transcriptionally regulated by the *daf* pathway, perhaps by DAF-2 or DAF-16. We have also observed DAF-12/GFP expression in hypodermal cells at the L2 and later stages, showing that *daf-12* is a stage specific gene activity in this tissue. This is consistent with  
15 the heterochronic effects of weak *daf-12* mutations.

This *daf-12*-GFP fusion also allowed us to view the dynamic regulation of *daf-12* gene action during insulin and TGF- $\beta$  regulated dauer or reproductive development. *daf-12* encodes a nuclear hormone receptor most closely related to the mammalian vitamin D and thyroid hormone receptors. We believe that the  
20 ligand for DAF-12 is likely to be regulated by insulin like or TGF- $\beta$  *daf* gene signaling. That ligand may be produced by the *C. elegans* equivalent to the thyroid gland, which may be related to the subesophageal glands of insects. For example, neurons in the retrovesicular ganglion of *C. elegans* may produce the *daf-12* ligand under DAF-16 and DAF-3 control. The mapping of exactly which

neurons the *daf-2* and *age-1* gene products function to regulate dauer arrest will identify the neuron. To identify the genes regulated by the DAF-16 and DAF-3 transcription factors, a yeast one hybrid experiment may be used (as described herein). GFP fusion to the genes so revealed should show that they are expressed in the key DAF-12 ligand producing neuron and are responsive to *daf-3* and *daf-16* mutations.

### *C. elegans*

In one working example, the above-described reporter gene construct is introduced into wild-type *C. elegans* according to standard methods known in the art. If the enhancer element is operational, then it is expected that reporter gene expression is turned on. Alternatively, in *daf* mutants (e.g., *daf-7* or *daf-2* mutants, where insulin signaling is defective) carrying the above-described reporter gene construct, reporter gene activity is turned off.

Using this on/off distinction, test compounds or extracts are evaluated for the ability to disrupt the signaling pathways described herein. In one working example, *daf-2* mutant worms carrying the reporter gene construct are used to assay the expression of the reporter gene. The majority of worms expressing the reporter gene will arrest at the dauer stage because of the *daf-2* phenotype. If however the test compound or extract inhibits DAF-16 activity, then the worms will exhibit a *daf-2*; *daf-16* phenotype (i.e., do not arrest), developing to produce eggs. Such eggs are selected using a bleach treatment and reporter gene expression in the test compound or extract is assayed according to standard methods, e.g., worms are examined with an automated fluorometer to reveal the presence of reporter gene expression, e.g., GFP. Candidate compounds that

suppress the *daf-2* phenotype or turn on reporter gene expression, i.e., activate signals in the absence of DAF-2 receptor (e.g., PIP3 mimetics) or inactivate DAF-16, are considered useful in the invention.

5 Analogous screens may also be performed using *daf-7* mutants as a means to identify drugs that inactivate other *daf*-genes, such as DAF-3, or compounds that activate the DAF-1/DAF-4 receptors. Such screens may be coupled to reporter screens, for example, using GFP reporter genes whose expression is under DAF-3 transcriptional control (e.g., the myoII element). Drugs identified in such screens are useful as DAF-7 mimetics. Because DAF-7  
10 expression may be down regulated in obesity, such drugs are useful in the treatment of obesity induced Type II diabetes

In yet another working example, *C. elegans* DAF-3 and DAF-16 genes can be replaced with a human homolog, (e.g., FKHR or FKHL1 for DAF-16),  
15 and screens similar to those described above performed in the nematode system. Because drugs may act upstream of the transcription factors, it is useful to replace DAF-1, DAF-4, DAF-8, DAF-14, DAF-2, DAF-3, DAF-16, or AGE-1 with the appropriate human homolog, and to screen the humanized *C. elegans* animals. Such screens are useful for identifying compounds having activities in humans.

## 20 **Mammalian Cells**

Mammalian insulin-responsive cell lines are also useful in the screening methods of the invention. Here reporter gene constructs (for example, those described above) are introduced into the cell line to evaluate the ability of a test compound or extract to modulate insulin and TGF- $\beta$  signaling pathways using a

second construct expressing a *C. elegans daf*, *age*, or *akt* gene or their corresponding human homologs. Exemplary cell lines include, but are not limited to, mouse 3T3, L6, and L1 cells (MacDougald et al., *Ann. Rev. Biochem.* 64: 345-373, 1995). Introduction of the constructs into such cell lines is carried out according to standard methods well known in the art.

To test a compound or extract, it is added to the cell line, and reporter gene expression is monitored. Compounds that induce reporter gene expression in the absence of insulin or DAF-7 signaling are considered useful in the invention. Such compounds may also turn the cells into adipocytes, as insulin does.

Compounds identified in mammalian cells may be tested in other screening assays described herein, and, in general, test compounds may be assayed in multiple screens to confirm involvement in insulin or DAF-7 signaling.

Metabolic control by DAF-7 protein may be tested using any known cell line, e.g., those described herein.

### **In Vitro Screening Methods**

A variety of methods are also available for identifying useful compounds in *in vitro* assays. In one particular example, test compounds are screened for the ability to activate the phosphorylation of Smad proteins, DAF-8, DAF-14, or DAF-3, by DAF-1 or DAF-4 *in vitro*. In these assays, DAF-8, DAF-14, or DAF-3 is preferably tagged with a heterologous protein domain, for example, the myc epitope tag domain(s) by the method of Ausubel et al., and are incubated with the C-terminal kinase domain of DAF-1 or DAF-4.

Phosphorylation of the Smad proteins is preferably detected by immunoprecipitation using antibodies specific to the tag, followed by scintillation

counting. Test compounds may be screened in high throughout microtiter plate assays. A test compound that effectively stimulates the phosphorylation of DAF-8, DAF-14, or DAF-3 is considered useful in the invention. Using these same general assays, compounds that activate the phosphorylation of DAF-16 by AKT or GSK-3 may also be identified.

In another working example, test compounds are screened for the ability to inhibit the *in vitro* association of DAF-16 and the Smad proteins DAF-3, or to preferentially activate the association of DAF-16 with DAF-8 or DAF-14, or to inhibit the association of DAF-3 and DAF-16 with DNA *in vitro*. These assays are carried out by any standard biochemical methods that test protein-protein binding or protein-DNA binding. In one particular example, to test for interactions between proteins, each protein is tagged with a different heterologous protein domain (as described above). Immunoprecipitations are carried out using an antibody to one tag, and an ELISA assay is carried out on the immunoprecipitation complex to test for the presence of the second tag. In addition, if interaction capability is enhanced by a DAF or AKT kinase, this protein is also preferably included in the reaction mixture. Similarly, to test for interactions of these proteins with DNA, antibodies to the tag are utilized in immunoprecipitations, and the presence of the DNA detected by the presence of the DNA label in the immunoprecipitation complex. A test compound that effectively inhibits the association between these proteins or between DAF-3 and DAF-16 with DNA or both is considered useful in the invention.

In still another working example, test derivatives of PIP3 are screened for the ability to increase *in vitro* AKT activity. This is accomplished, in general, by combining a labeled PIP3 and an AKT polypeptide in the presence and absence

of the test compound under conditions that allow PIP3:AKT to bind *in vitro*.

Compounds are then identified that interfere with the formation of the PIP3:AKT complex. Test compounds that pass this first screen may then be tested for increased AKT activation *in vitro* using GSK3 targets, or may be tested in

5 nematodes or mammalian cells (as described above). An increase in AKT kinase activity is taken as an indication of a compound useful for ameliorating or delaying an impaired glucose tolerance condition.

In yet another working example, DAF-3 or DAF-16 may be expressed in a yeast one-hybrid assay for transcriptional activation. Methods for such assays  
10 are described in Brent and Ptashne (*Cell* 43:729-736, 1985). A test compound that blocks the ability of DAF-3 or DAF-16 or both to activate (or repress) transcription in this system is considered useful in the invention.

In a final working example, compounds may be screened for their ability to inhibit an interaction between any of DAF-3, DAF-8, and DAF-14, or between  
15 DAF-3 and DAF-16. These *in vivo* assays may be carried out by any “two-hybrid” or “interaction trap” method (for example, by using the methods described by Vijaychander et al (*Biotechniques* 20: 564-568)).

### **Screens for Isolating Longevity Therapeutics**

The worm insulin signaling pathway has been implicated in longevity  
20 control of *C. elegans*. Drugs which perturb this pathway could affect lifespan. Specifically, inhibition of the pathway would be expected to extend lifespan. Drugs that inactivate the DAF-2 ligands, the AGE-1 PI3 kinase, or decrease PIP3 signals in any way, for example, by increasing DAF-18/PTEN activity, decreasing PDK or AKT activity, or decreasing the phosphorylation of DAF-16, are expected

to increase longevity. Such drugs may be used topically on the skin to increase longevity in this organ. It is significant that AGE-1 generates a second messenger, PIP3, that directly regulates AKT and perhaps PDK activity.

Antagonists to PIP3 are expected to extend lifespan, but any drug that mimics the activity state of the pathway during aging is expected to increase longevity. For example, drugs causing low activity of the following proteins: DAF-2 agonist, DAF-2 receptor, AGE-1, PDK, and AKT, would increase longevity. Drugs causing high activity of PTEN or DAF-16 (high meaning unphosphorylated) would increase longevity.

The insulin-like signaling genes that function in metabolic regulation and molting control also function to control aging in the animal. We have shown that declines in *daf-2* insulin receptor-like *age-1* PI-3 kinase, PDK-1, and akt-1/2 signaling cause dauer arrest and a corresponding increase in lifespan and a change in metabolism towards fat storage. Thus, drugs that perturb the gene activities in this pathway are expected to regulate longevity as well as metabolism. Specifically, chemicals that decrease the activity of the human homologues of the DAF-2 insulin/IGF-I receptor homologue, decrease the activity of the human homologue of the AGE-1/PI3 kinase, decrease the activity of human homologues of AKT-1 and AKT-2, decrease the activity of the human homologue of PDK-1, or inhibit the phosphorylation of the human homologues of DAF-16 by the human homologues of AKT-1 and AKT-2 increase longevity. Chemicals that increase the activity of DAF-18 PTEN also increase longevity, since decreases in DAF-18 activity decrease longevity.

Similarly, the AGE-1 and AKT-1/2 proteins are enzymes with in vitro activities. An AGE-1 assay preferably involves phosphorylation of a phosphatidyl

inositol target on the 3 position. The AKT-1 or AKT-2 kinase assay involves phosphorylation of DAF-16 as well as the human DAF-16 homologues, FKHR, FKHL1, and AFX targets. Chemical screens for drugs that inhibit in vitro activities of the human homologues of these *C. elegans* kinases are first preferably performed in vitro. Chemicals that perturb this function are then tested on *C. elegans* mutants carrying the human gene as the only functional copy of the gene. If desired, positive drugs could then be tested on mice for those that increase longevity.

### **Screens for Identifying Pesticide and Nematicide Compounds**

Our discovery that converging insulin-like and DAF-7 TGF- $\beta$  like neuroendocrine signals regulate diapause arrest in *C. elegans* is also important for the development of novel nematicides and pesticides. For example, the finding that insulin like signaling regulates metabolism in animals as phylogenetically distant as nematodes and mammals suggests that this pathway was present in the common ancestor of worms and mammals over 600,000,000 years ago. Diapause, the suspension of development by environmental signals, is phyletically general. In view of the results described herein, insulin may regulate diapause/developmental arrest in many animals, including insects and other nematodes. In fact, human insulin induces recovery of diapausing corn borers, and a cholinergic neuronal input to dauer arrest has been shown herein to exist in both *C. elegans* and the mammalian parasitic nematode *Ancylostoma caninum*. These observations indicate that daf pathway results from *C. elegans* can be generalized to distant nematode relatives as well as other invertebrates, most importantly, insects. Since diapause is a non feeding state, novel insecticides and nematicides may be

developed which induce diapause if the insulin like pathway can be inactivated in insects or nematodes. Specifically, drugs that induce downregulation of the insect or parasitic nematode homologues of DAF-2, AGE-1, PDK-1, AKT-1, or AKT-2, or upregulation of DAF-18 or DAF-16 would induce non feeding diapause. Such an agent would be expected to protect crops from destruction by feeding and infection. In addition, agents that induce activity of DAF-2, AGE-1, PDK-1, AKT-1, or AKT-2, or downregulation of DAF-18 or DAF-16 would be expected to induce recovery from diapause. Since diapause is an overwintering stress resistant state, and is generally the infective stage of plant and animal parasitic nematodes, such agents would improve pest infestations by perturbing the overwintering or infective process.

### **Modulatory Compounds**

Our experimental results facilitate the isolation of compounds useful in the treatment of impaired glucose tolerance diseases that are antagonists or agonists of the insulin or TGF- $\beta$  signaling pathways identified in *C. elegans* and described above. Exemplary methods for the isolation of such compounds now follow.

### **Antagonists**

As discussed above, useful therapeutic compounds include those which down regulate the expression or activity of DAF-3, DAF-16, or DAF-18 (PTEN). To isolate such compounds, DAF-3, DAF-16, or DAF-18 (PTEN) expression is measured following the addition of candidate antagonist molecules to a culture medium of DAF-3, DAF-16, or DAF-18 (PTEN) expressing cells. Alternatively,

the candidate antagonists may be directly administered to animals (for example, nematodes or mice) and used to screen for their effects on DAF-3, DAF-16, or DAF-18 (PTEN) expression.

DAF-3, DAF-16, or DAF-18 (PTEN) expression is measured, for example, by standard Northern blot analysis (Ausubel et al., *supra*) using a DAF-3, DAF-16, or DAF-18 (PTEN) nucleic acid sequence (or fragment thereof) as a hybridization probe. The level of DAF-3, DAF-16, or DAF-18 (PTEN) expression in the presence of the candidate molecule is compared to the level measured for the same cells, in the same culture medium, or in a parallel set of test animals, but in the absence of the candidate molecule. Preferred modulators for anti-diabetic or anti-obesity purposes are those which cause a decrease in DAF-3, DAF-16, or DAF-18 (PTEN) expression.

Alternatively, the effect of candidate modulators on expression or activity may be measured at the level of DAF-3, DAF-16, or DAF-18 (PTEN) protein production using the same general approach in combination with standard immunological detection techniques, such as Western blotting or immunoprecipitation with a DAF-3, DAF-16, or DAF-18 (PTEN) specific antibody (for example, the DAF-3 or DAF-16 antibodies described herein). Again, useful anti-diabetic or anti-obesity therapeutic modulators are identified as those which produce a decrease in DAF-3, DAF-16, or DAF-18 (PTEN) polypeptide production. Antagonists may also affect DAF-3, DAF-16, or DAF-18 (PTEN) activity without any effect on expression level. For example, the identification of kinase cascades upstream of DAF-3 and DAF-16 (as described herein) suggest that the phosphorylation state of these polypeptides is correlated with activity. Phosphorylation state may be monitored by standard Western blotting using

antibodies specific for phosphorylated amino acids. In addition, because DAF-3 and DAF-16 are transcription factors, reporter genes bearing operably linked DAF-3 or DAF-16 binding sites (for example, the myoII enhancer element) may be used to directly monitor the effects of antagonists on DAF-3 or DAF-16 gene activity.

5           Candidate modulators may be purified (or substantially purified) molecules or may be one component of a mixture of compounds (e.g., an extract or supernatant obtained from cells). In a mixed compound assay, DAF-3, DAF-16, or DAF-18 (PTEN) expression is tested against progressively smaller subsets of the candidate compound pool (e.g., produced by standard purification techniques, e.g.,  
10   HPLC or FPLC; Ausubel et al., *supra*) until a single compound or minimal compound mixture is demonstrated to modulate DAF-3, DAF-16, or DAF-18 (PTEN) expression.

          Candidate DAF-3, DAF-16, or DAF-18 (PTEN) antagonists include peptide as well as non-peptide molecules (e.g., peptide or non-peptide molecules  
15   found, e.g., in a cell extract, mammalian serum, or growth medium on which mammalian cells have been cultured).

          Antagonists found to be effective at the level of cellular DAF-3, DAF-16, or DAF-18 (PTEN) expression or activity may be confirmed as useful in animal models (for example, nematodes or mice). For example, the compound  
20   may ameliorate the glucose intolerance and diabetic symptoms of mouse models for Type II diabetes (e.g., a db mouse model), mouse models for Type I diabetes, or models of streptozocin- induced  $\beta$  cell destruction.

          A molecule which promotes a decrease in DAF-3, DAF-16, or DAF-18 (PTEN) expression or DAF-3, DAF-16, or DAF-18 (PTEN) activity is considered  
25   particularly useful in the invention; such a molecule may be used, for example, as

a therapeutic to decrease the level or activity of native, cellular DAF-3, DAF-16, or DAF-18 (PTEN) and thereby treat a glucose intolerance condition in an animal (for example, a human).

5 If desired, treatment with an antagonist of the invention may be combined with any other anti-diabetic or anti-obesity therapies.

### Agonists

Also as discussed above, useful therapeutic compounds are those which up regulate the expression or activity of DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1. To isolate such compounds,  
10 expression of these genes is measured following the addition of candidate agonist molecules to a culture medium of DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 expressing cells. Alternatively, the candidate agonists may be directly administered to animals (for example, nematodes or mice) and used to screen for effects on DAF-1, DAF-2, DAF-4,  
15 DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 expression.

DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 expression is measured, for example, by standard Northern blot analysis (Ausubel et al., *supra*) using all or a portion of one of these genes as a hybridization probe. The level of DAF-1, DAF-2, DAF-4, DAF-7,  
20 DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 expression in the presence of the candidate molecule is compared to the level measured for the same cells, in the same culture medium, or in a parallel set of test animals, but in the absence of the candidate molecule. Preferred modulators for anti-diabetic or anti-obesity purposes are those which cause an increase in DAF-1, DAF-2, DAF-4,

DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 expression.

Alternatively, the effect of candidate modulators on expression may be measured at the level of DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5 or PDK-1 protein production using the same general approach in combination with standard immunological detection techniques, such as Western blotting or immunoprecipitation with an appropriate antibody. Again, the phosphorylation state of these polypeptides is indicative of DAF activity and may be measured on Western blots. Useful anti-diabetic or anti-obesity modulators are identified as those which produce an increase in DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 polypeptide production.

Candidate modulators may be purified (or substantially purified) molecules or may be one component of a mixture of compounds (e.g., an extract or supernatant obtained from cells). In a mixed compound assay, DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 expression is tested against progressively smaller subsets of the candidate compound pool (e.g., produced by standard purification techniques, e.g., HPLC or FPLC; Ausubel et al., *supra*) until a single compound or minimal compound mixture is demonstrated to modulate DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 expression.

Alternatively, or in addition, candidate compounds may be screened for those which agonize native or recombinant DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 activities. In one particular example, DAF-1 and DAF-4 phosphorylation of DAF-8 and DAF-14, or AKT phosphorylation of DAF-16, may be activated by agonists.

Candidate DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 agonists include peptide as well as non-peptide molecules (e.g., peptide or non-peptide molecules found, e.g., in a cell extract, mammalian serum, or growth medium on which mammalian cells have been cultured).

Agonists found to be effective at the level of cellular DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 expression or activity may be confirmed as useful in animal models (for example, nematodes or mice).

A molecule which promotes an increase in DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 expression or activities is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to increase the level or activity of these native, cellular genes and thereby treat a glucose intolerance condition.

If desired, treatment with an DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, AKT, COD-5, or PDK-1 agonist may be combined with any other anti-diabetic or anti-obesity therapies.

### **Animal Model Systems**

Compounds identified as having activity in any of the above-described assays are subsequently screened in any number of available diabetic or obesity animal model systems, including, but not limited to ob (Coleman, *Diabetologia* 14: 141-148, 1978; Chua et al., *Science* 271: 994-996, 1996; Vaisse et al., *Nature Genet.* 14:95-100, 1996), db (Chen et al., *Cell* 84: 491-495, 1996), agouti mice, or fatty rats (Takaga et al. *Biochem. Biophys. Res. Comm.* 225: 75-83, 1996). Test

compounds are administered to these animals according to standard methods. Additionally, test compounds may be tested in mice bearing knockout mutations in the insulin receptor, IGF-1 receptor (e.g., Liu et al., Cell 75:59-72, 1993), IR-related receptor, DAF-7 homolog, or any of the *daf* (FKHR, FKHL1, AFX) genes described herein. Compounds can also be tested using any standard mouse or rat model of Type I diabetes, e.g., a streptozin ablated pancreas model.

In one particular example, the invention involves the administration of DAF-7 or its homolog as a method for treating diabetes or obesity. Evaluation of the effectiveness of such a compound is accomplished using any standard animal model, for example, the animal diabetic model systems described above. Because these mouse diabetic models are also associated with obesity, they provide preferred models for human obesity associated Type II diabetes as well. Such diabetic or obese mice are administered *C. elegans* or human DAF-7 according to standard methods well known in the art. Treated and untreated controls are then monitored for the ability of the compound to ameliorate the symptoms of the disease, e.g., by monitoring blood glucose, ketoacidosis, and atherosclerosis. Normalization of blood glucose and insulin levels is taken as an indication that the compound is effective at treating a glucose intolerance condition.

### Therapy

Compounds of the invention, including but not limited to, DAF-7 and its homologs, and any antagonist or agonist therapeutic agent identified using any of the methods disclosed herein, may be administered with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or

compositions to administer such compositions to patients. Although intravenous administration is preferred, any appropriate route of administration may be employed, for example, parenteral, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for antagonists or agonists of the invention include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

DAF polypeptides are administered at any appropriate concentration, for example, for DAF-7, at a concentration of around 10nM.

#### **Pedigree Analysis and Genetic Testing**

Abstract

The discovery described herein that DAF polypeptides are involved in glucose metabolism enables assays for genetic testing to identify those individuals with predispositions toward the development of glucose intolerance conditions, such as diabetes or obesity, by determining the presence of a mutation found in a human gene having identity to any of the *C. elegans daf-1, daf-2, daf-3, daf-4, daf-7, daf-8, daf-11, daf-14, daf-16, age-1, akt, daf-18* (PTEN), or *pdk-1* genes. In one embodiment, the development of this testing method requires that the individual be a member of a family that has multiple affected and unaffected members carrying one mutation from the list of above-listed genes. Those skilled in the art will understand that a diabetic or obesity phenotype may be produced by *daf, age, or akt* mutations found on different chromosomes, and that low resolution genetic mapping of the diabetic condition in single family pedigrees will be sufficient to favor some *daf, age, or akt* genes over others as causing the glucose intolerance condition in a particular pedigree. In one particular example, mutations associated with glucose intolerance may be found in different genes in, for example, the DAF-7 signaling pathway in each pedigree. In addition, because mutations in a common pathway can show complex genetic interactions, multiple DAF mutations may segregate in single pedigree. These mutations can behave recessively in some genetic backgrounds and dominantly in others.

Those skilled in the art further understand that, to determine disease linkage with a chromosomal marker, it may be necessary to evaluate the association of inheritance patterns of several different chromosomal markers (for example, from the collection of highly polymorphic mapped DNA allelic variants) in the genomic DNAs of family members and of the clinically affected individuals. Methods commonly used in determining segregation patterns of human genetic

diseases are well known in the art. In addition, methods are known in the art for determining whether individuals in a family are useful for providing information to determine co-segregation of an allele with a glucose intolerance trait.

Here, fragments of genomic DNA (e.g., RFLP fragments) are prepared from each of the available members of the family, and each distinctive DNA allelic variant of the polymorphic chromosome marker within the family is evaluated to determine which polymorphisms (i.e., chromosomal region) is linked with the glucose intolerance phenotype within a particular family. It is preferred that the parents of the marker individual be heterozygous for a DNA allelic variant so that the segregation pattern of the DNA allelic variant linked with the diabetic or obese phenotype in the marker can be recognized. The inheritance of the diabetic phenotype can be judged to be dominant or recessive, depending on the pattern of inheritance. Most diabetes is dominantly inherited, and therefore inbred pedigrees are generally not necessary in the etiology of the diabetic condition.

With respect to Type II diabetes, the highest rate of this kind of diabetes in the world is found in American Indians of the Pima tribe. Such families are useful for mapping one particular cause of diabetes, but, in general, diabetes is caused by mutations in a variety of genes, including *daf* genes. Thus, by testing for low resolution linkage to a candidate *daf*, *age*, or *akt* mutation, and then by sequencing the particular linked *daf* gene in affected and unaffected individuals, a particular *daf* mutation can be associated with a particular diabetic pedigree.

Human DAF homologues are mapped to chromosome regions using standard methods. For example, the probable DAF-16 homologues FKHR and FKHL1 are located on chromosomes 13 and 11, respectively, and AFX is located on the X chromosome. In particular, candidate loci for human DAF homologues

are as follows: P85=5q13, P110alpha=3q26.3, PTEN=10q23.3, Akt-1=14q32.3, Akt-2=19q13.1, FKHL1=11q23, FKHR=13q14.1, Afx=xq13.1, and Daf-7 (GDF-8)=2q32.1 (the position at which NIDDM1 has been mapped).

Any *daf*, *akt*, or *age* genes mapping to the approximate chromosomal regions associated with diabetes or glucose intolerance are sequenced from affected and unaffected individuals. Preferably, at least two genes per pedigree of 5-20 affected (and unaffected controls) are sequenced. The *daf* genomic regions are PCR amplified and compared between affected and unaffected DNA samples. Mutations detected in affected individuals are expected to (but need not) map to conserved domains of the DAF genes. Because it is known that not all carriers of known diabetes-inducing mutations show metabolic defects, we expect that some non-diabetic non-glucose intolerant family members will carry the same *daf* mutation as affected family members. For this reason, a correlation of affected family members with a *daf* mutation is more important than a correlation of nonaffected with no mutation. Those skilled in the art will know that phenotypic classification of affected and unaffected individuals can greatly enhance the power of this genetic analysis (Nature Genet. 11: 241-247, 1995). In addition, other mutations in the same *daf* gene are expected in some but not all diabetic pedigrees. For dominant diabetic inheritance, the affected individuals carry a *daf*, *age*, or *akt* mutation as well as a normal allele. For recessive diabetic inheritance, individuals carry two *daf* mutations that may be identical or two independent mutations in the same gene. In addition, some diabetic individuals may carry mutations in more than one *daf*, *age*, or *akt* gene (so called non-allelic non-complementation).

It is routine in the art of genetic counseling to determine risk factors given the presence of a closely linked molecular genetic marker in the genomic

DNA of the individual and when combined with the additional understanding provided by the pedigree of the individual in the family. For example, a risk factor may be calculated for an individual in an *age*, *akt*, or *daf* chromosome family in a manner similar to those described for assessing the risk of other commonly known genetic diseases that are known to run in families, e.g., Huntington's disease and cystic fibrosis.

Once mutations in *daf*, *akt*, or *age* genes are associated with diabetes in a pedigree analysis, diagnostic PCR sequencing of these *daf* genes can be used to diagnose glucose intolerant, prediabetic, diabetic, obesity, and atherosclerotic conditions. Preferably, the *daf*, *akt*, or *age* gene regions are PCR amplified from patients and mutations detected in the *daf* genes using standard DNA sequencing or oligonucleotide hybridization techniques. The use of such gene sequences or specific antibody probes to the products of these sequences provide valuable diagnostics, particularly in view of the likelihood there exist two classes of type II diabetics: those with defects in the TGF- $\beta$  signaling genes, and those with defects in insulin signaling genes. Such genetic tests will influence whether drugs that affect DAF-7 TGF- $\beta$  or DAF-2 insulin like signals are prescribed.

To carry out the above analysis (as well as the other screening, diagnostic, and therapeutic methods described herein), mammalian homologs corresponding to the *C. elegans* *daf-1*, *age-1*, *daf-4*, *daf-8*, and *daf-7* genes are isolated as described above for *daf-2*, *daf-3*, and *daf-16*. Again, standard hybridization or PCR cloning strategies are employed, preferably utilizing conserved DAF, AGE, or AKT motifs for probe design followed by comparison of less conserved sequences flanking these motifs. Exemplary motifs for these genes are as follows:

DAF-1 (139 amino acid motif) (SEQ ID NO: 13)

274 TSGSGMGPTTLHKL TIGGQIRLTGRVGSGRFGNVSRGDYRGEAVAVK  
VFNALDEPAFHKETE I FETRMLRHPNVLR YIGSDRVD TG FVT ELWL VTEYH  
PSGSLHDFLENTVNI E TYYNLMRSTASGLA FLHNQIGGSK 412

5 DAF-1 (62 amino acid motif) (SEQ ID NO: 14)

450 EDAASDIANENYKCGTVRYLAPEILNSTMQFTVFESYQCADVYSFSL  
VMWETLCRCEDGDV 511

DAF-1 (31 amino acid motif) (SEQ ID NO: 15)

416 KPAMAH<sup>RD</sup>IKSKNIMVKN<sup>DL</sup>TCAIGDLGLSL 466

10 DAF-1 (72 amino acid motif) (SEQ ID NO: 16)

520 IPYIEWTDRDPQDAQMFDVVCTRRLRPTENPLWKDHPMKHIMEIIKT  
CWNGNPSARFTS YICRKRMDERQQ 591

AGE-1 (150 amino acid motif) (SEQ ID NO: 17)

991 YFESVDRFLYSCVGYSVATYIMGIKDRHSDNMLTEDGKYVHIDFGHI  
15 LGHGKTKLGIQRDRQPFI L TEHFMTVIRSGKSVDGNSHELQKFKTL C VEAY  
EVMWNNRDLFVSLFTLMLGMELPELSTKADLDHLKKTLFCNGESKEEAR  
KF 1140

AGE-1 (113 amino acid motif) (SEQ ID NO: 18)

826 SPLDPVYKLGEMIIDKAIVLGSKRPLMLHWKNKNPKSDLHLPCAMI

FKNGDDLRLQDMLVLQVLEVMDNIWKAANIDCCLNPYAVLPMGEMIGIIE  
VVPNCKTIFEIQVGTG 938

AGE-1 (106 amino acid motif) (SEQ ID NO: 19)

642 LAFVWTDRENFSELYVMLEKWPPSVAAALTLLGKRCTDRVIRKFAV  
5 EKLNEQLSPVTFHLFILPLIQALKYEPRAQSEVGMMLLTRALCDYRIGHRLF  
WLLRAEI 747

AGE-1 (60 amino acid motif) (SEQ ID NO: 38)

91 EIKLSDFKHQLFELIAPMKWGTYSVKPQDYVFRQLNMFGEIEVIFND  
DQPLSKLELHGTF 150

10 AKT (121 amino acid motif) (SEQ ID NO: 60)

33685 QVLDDHDYGRCDVWWGVGVVMYEMMCGRLPFYSKDHKNLF  
ELIMAGDLRFPSKLSQEARTLLTGLLVKDPTQRLGGGPEDALEICRADFFR  
TVDWEATYRKEIEPPYKPNVQSETDTSYFD 34047

AKT (66 amino acid motif) (SEQ ID NO: 61)

15 32314 TMEDFDFLKVLGKGTFGKVILCKEKRTQKLYAIKILKKDVIIARE  
EVAHTLTENRVLQRCKHPFLT 32511

AKT (45 amino acid motif) (SEQ ID NO: 62)

33509 KLENLLLDKDGHIKIADFGLCKEEISFGDKTSTFCGTPEYL  
APEV 33643

AKT (57 amino acid motif) (SEQ ID NO: 63)

32667 YFQELKYSFQEQHYLCFVMQFANGGELFTHVRKCGTFSEPRARFY  
GAEIVLALGYLH 32837

AKT (59 amino acid motif) (SEQ ID NO: 64)

5 31846 STFAIFYFQTMLFEKPRPNMFMVRCLQWTTVIERTFYAESAEVRQ  
RWIHAIESISKKYK 32022

AKT (33 amino acid motif) (SEQ ID NO: 65)

33156 LQELKYSFQTNDRLCFVMEFAIGGDLYYHLNRE 33254

AKT (21 amino acid motif) (SEQ ID NO: 66)

10 30836 VVIEGWLHKKGEHIRNWRPRF 30898

AKT (26 amino acid motif) (SEQ ID NO: 67)

33276 FSEPRARFYGSEIVLALGYLHANSIV 33353

DAF-4 (139 amino acid motif) (SEQ ID NO: 20)

380 EYWIVTEFHERLSLYELLKNNVISITSANRIIMSMIDGLQFLHDDRPFYFF  
15 GHPKKPIIHRDIKSKNILVKSDMTTCIADFGLARIYSYDIEQSDLLGQVGTK  
RYMSPEMLEGATEFTPTAFKAMDVYSMGLVMWEVISR 518

DAF-4 (61 amino acid motif) (SEQ ID NO: 21)

537 IGFDP TIGRMRNYVVSKKERPQWRDEIIKHEYMSLLKKVTEEMWDPE  
ACARITAGCAFARV 597

DAF-4 (20 amino acid motif) (SEQ ID NO: 22)

305 PITDFQLISKGRFGKVFKAQ 324

DAF-8 (163 amino acid motif) (SEQ ID NO: 23)

382 TDSETRSRFSLGWYNNPNRSPQTAEVRLIGKGVRFYLLAGEVYVENL

5 CNIPVVFVQSIGANMKNGFQLNTVSKLPPTGTMKVFDMLFSKQLRTAAEK

TYQDVYCLSRMCTVRVSECKGWGEHYRRSTVLRSPVWFQAHLNNPMHW

VDSVLTCMGAPPRICSS 544

DAF-8 (44 amino acid motif) (SEQ ID NO: 24)

91 RAFRFPVIRYESQVKSILTCRHAFNSHSRNVCLNPYHYRWVELP 134

10 DAF-8 (38 amino acid motif) (SEQ ID NO: 25)

341 VEYEESPSWLKLIYYEEGTMIGEKADEVGGHHCLIDGFT 378

DAF-14 (39 amino acid motif) (SEQ ID NO: 68)

9709 IRVSFCKGFGETYSLKVVNLPCWIEILHEPADEYDTV 9825

DAF-14 (45 amino acid motif) (SEQ ID NO: 69)

15 9409 SRNSKSSQIRNTVGAGIQLAYENGELWLTVLTQIVFVQCPFLNQ

9543

DAF-14 (29 amino acid motif) (SEQ ID NO: 70)

9160 NEMLDPEPKYPKEEKPWCTIFYYEELTVRV 9246

DAF-14 (29 amino acid motif) (SEQ ID NO: 71)

9307 QL GK AFEAKVPTITIDGATGASDECRMSL 9393

DAF-12 (105 amino acid motif) (SEQ ID NO: 72)

103 SPDDGLLDSSEESRRRQKTCRVCGDHATGYNFNVITCESCKAFFRR

5 NALRPKEFKCPYSEDCEINSVSRRFCQKCRLRKCF TVGMKKEWILNEEQLR  
RRKNSRLN 207

DAF-12 (89 amino acid motif) (SEQ ID NO: 73)

109 LDSSEESRRRQKTCRVCGDHATGYNFNVITCESCKAFFRRNALRPKE

10 FKCPYSEDCEINSVSRRFCQKCRLRKCF TVGMKKEWILNEEQ 197

DAF-12 (73 amino acid motif) (SEQ ID NO: 74)

551 DIMNIMDV TMRRFVKVAKGVPAFREVSQEGKFSLKGGMIEMLT V

RGVTRYDASTNSFKTPTIKGQNVSVNVD 623

DAF-11 (112 amino acid motif) (SEQ ID NO: 75)

15 708 SGSLVDLMIKNLTAYTQGLNETVKNRTAELEKEQEKG DQLLMELL

PKSVANDLKN GIAVDPKVYENATILYSDIVGFTSLCSQS QPMEVVTLLSGM

YQRFDLIISQQGGYKV 819

DAF-11 (107 amino acid motif) (SEQ ID NO: 76)

825 METIGDAYCVAAGLPV VMEKDHVKSICMIAL LQRDCLHHFEIPHR

20 PGTFLNCRWGFNSGPVFAGVIGQKAPRYACFGEAVILASKMESSGVEDRIQ

MTLASQQLLEE 931

DAF-11 (43 amino acid motif) (SEQ ID NO: 77)

520 DILKGGLEYIHASAIIDFHGNLTLHNCMLDSHWIVKLSGFGVNRL 562

DAF-11 (15 amino acid motif) (SEQ ID NO: 78)

618 DMYSFGVILHEILK 632

5 DAF-7 (60 amino acid motif) (SEQ ID NO: 26)

290 NLAETGHSKIMRAAAHKVSNPEIGYCCHPTEYDYIKLIYVNRDGRVSIA  
NVNGMIAKKCGC 349

DAF-7 (20 amino acid motif) (SEQ ID NO: 27)

265 DWIVAPPRYNAYMCRGDCHY 284

10 DAF-7 (43 amino acid motif) (SEQ ID NO: 28)

240 VCNAEAQSKGCCLYDLEIEFEKIGWDWIVAPPRYNAYMCRGDC 282

DAF-7 (70 amino acid motif) (SEQ ID NO: 29)

281 DCHYNAHHFNLAETGHSKIMRAAAHKVSNPEIGYCCHPTEYDYIKLIYV  
NRDGRVSIANVN GMIAKKCGCS 350

15 DAF-7 (35 amino acid motif) (SEQ ID NO: 30)

250 CCLYDLEIEFEKIGWDWIVAPPRYNAYMCRGDCHY 284

DAF-7 (13 amino acid motif) (SEQ ID NO: 51)

GWDWIVAPPRYNA

DAF-7 (9 amino acid motif) (SEQ ID NO: 364)

GWDXXIAPK

In one particular example, mammalian DAF-7 may be identified using the sub-domain amino acids 314-323. Exemplary degenerate oligonucleotides  
5 designed to PCR amplify this domain or hybridize (for example, as described in Burglin et al., (Nature 341:239-243, 1989) are as follows:

aa 263 oligo: GGNTGGGAYTRNRTNRTNGCNCC (23-mer, 16,000-fold degeneracy) (SEQ ID NO: 31)

aa 314 oligo: TGYTGYNNNCCNACNGAR (18-mer, 8000-fold  
10 degeneracy) (SEQ ID NO: 32).

The DNA sequence between the oligonucleotide probes is determined, and those sequences having the highest degree of homology are selected. Once isolated, these sequences are then tested in a *C. elegans daf-7* mutant or mouse model as described above for the ability to functionally complement the mutation  
15 or ameliorate the glucose intolerance phenotype.

To date, the closest homologues of *C. elegans* appear to be members of the vertebrate GDF-8 and GDF-11 gene family, with a representative homologue shown in Figures 47A and 47B. These human proteins, whose composition and function in muscle size determination have been described (McPherron AC,  
20 Lee SJ, Proc Natl Acad Sci U.S.A.1997 Nov 11;94(23):12457-61), may also function in metabolic control in conjunction with insulin. Alternatively, there may be more than one DAF-7 orthologue, or a closer relative to DAF-7 in

mammalian databases that subserves the metabolic role, whereas GDF-8,11 serve related roles in muscle control. The DAF-7 gene does not appear in worm EST databases, most likely because it is expressed in a single neuron, a very low expression level. Even though the mammalian EST databases are about 10 fold larger than the *C. elegans* EST base, if human DAF-7 is expressed in a small set of neurons, it is not surprising that it has not yet been seen in the EST database. Nonetheless, human DAF-7 may be instantly recognized using the motif, GWDXXIAPK as a means to search updated sequence databases or by standard techniques as described herein.

#### Other Embodiments

In other embodiments, the invention includes any protein which possesses the requisite level of amino acid sequence identity (as defined herein) to DAF-2, DAF-3, or a DAF-16 sequence; such homologs include other substantially pure naturally-occurring mammalian DAF polypeptides (for example, human DAF polypeptides) as well as allelic variants; natural mutants; induced mutants; proteins encoded by DNA that hybridizes to the DAF DNA sequence or degenerate conserved domains of DAF proteins (e.g., those described herein) under high stringency conditions; and proteins specifically bound by antisera directed to a DAF-2, DAF-3, or DAF-16 polypeptide.

The invention further includes analogs of any naturally-occurring DAF-2, DAF-3, or DAF-16 polypeptides. Analogs can differ from the naturally-occurring protein by amino acid sequence differences which do not destroy function, by post-translational modifications, or by both. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, e.g., acetylation,

carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring DAF polypeptide by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, 1989, or Ausubel et al., *supra*). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g.,  $\beta$  or  $\gamma$  amino acids.

In addition to full-length polypeptides, the invention also includes DAF-2, DAF-3, and DAF-16 polypeptide fragments. As used herein, the term "fragment," means at least 20 contiguous amino acids, preferably at least 30 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of such DAF polypeptides can be generated by methods known to those skilled in the art or may result from normal protein processing (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

For certain purposes, all or a portion of the DAF-2, DAF-3, or DAF-16 polypeptide sequence may be fused to another protein (for example, by recombinant means). In one example, the DAF polypeptide may be fused to the green fluorescent protein, GFP (Chalfie et al., *Science* 263:802-805, 1994). Such a

fusion protein is useful, for example, for monitoring the expression level of the DAF polypeptide *in vivo* (for example, by fluorescence microscopy) following treatment with candidate or known DAF agonists or antagonists.

5 The methods of the invention may be used to diagnose or treat any condition related to glucose intolerance or obesity in any mammal, for example, humans, domestic pets, or livestock. Where a non-human mammal is diagnosed or treated, the DAF polypeptide, nucleic acid, or antibody employed is preferably specific for that species.

10 All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

Other embodiments are within the following claims.

What is claimed is:

## Claims

1. A method for identifying a compound that modulates the expression or activity of a *daf-18* gene, comprising:

(a) providing a cell expressing a *daf-18* gene; and

5 (b) contacting said cell with a candidate compound, an alteration in *daf-18* expression or activity following contact with said candidate compound identifying a modulatory compound.

2. A method for identifying a compound that modulates the expression or activity of a *daf-18* gene, comprising:

10 (a) providing a cell comprising a mutation in a *daf-18* gene;

(b) expressing in said cell a mammalian DAF-18 homologue; and

(c) contacting said cell with a candidate compound, an alteration in said mammalian DAF-18 expression or activity following contact with said candidate compound identifying a modulatory compound.

15 2. The method of claim 1 or 2, wherein said compound increases *daf-18* expression or activity and is therefore capable of increasing longevity of a cell or organism.

20 3. The method of claim 1 or 2, wherein said compound decreases *daf-18* activity and is capable of treating an impaired glucose tolerance condition or obesity.

4. The method of claim 1 or 2, wherein said method is carried out in a transgenic animal.

5. The method of claim 4, wherein said animal is a nematode.

6. The method of claim 4, wherein said animal is a mouse.

5 7. The method of claim 2, wherein said DAF-18 homologue is a human homologue.

8. The method of claim 7, wherein said DAF-18 homologue is PTEN.

9. A method for identifying a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition or obesity, comprising  
10 contacting a biological sample with a candidate compound and assaying said sample for DAF-18-mediated lipid phosphatase activity, a decrease in said activity indicating a compound capable of ameliorating or delaying an impaired glucose tolerance condition or obesity.

10. A method for identifying a compound that is capable of increasing  
15 longevity of a cell or organism, comprising contacting a biological sample with a candidate compound and assaying said sample for DAF-18-mediated lipid phosphatase activity, an increase in said activity indicating a compound capable of increasing longevity of a cell or organism.

11. A method for identifying a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition or obesity, comprising contacting a biological sample with a candidate compound and assaying said sample for PTEN-mediated lipid phosphatase activity, a decrease in  
5 said activity indicating a compound capable of ameliorating or delaying an impaired glucose tolerance condition or obesity.

12. A method for identifying a compound that is capable of increasing longevity of a cell or organism, comprising contacting a biological sample with a candidate compound and assaying said sample for PTEN-mediated lipid  
10 phosphatase activity, an increase in said activity indicating a compound capable of increasing longevity of a cell or organism.

13. The method of claim 9 or 11, wherein said method further comprises assaying said compound in a cell which comprises a mutation in a *daf-18* gene and which expresses a mammalian DAF-18 homologue, a decrease in  
15 DAF-18 activity indicating a compound capable of treating an impaired glucose tolerance condition or obesity.

14. The method of claim 10 or 12, wherein said method further comprises assaying said compound in a cell which comprises a mutation in a *daf-18* gene and which expresses a mammalian DAF-18 homologue, an increase in  
20 DAF-18 activity indicating a compound capable of increasing longevity of a cell or organism.

15. The method of claim 13, wherein said mammalian DAF-18 homologue is human PTEN.

16. The method of claim 14, wherein said mammalian DAF-18 homologue is human PTEN.

5           17. A method of diagnosing an impaired glucose tolerance condition, obesity, or a propensity thereto in a patient, said method comprising analyzing the level of PTEN expression or activity in a sample isolated from said patient, whereby an increase in said level of PTEN expression or activity relative to a control sample is an indication of an impaired glucose tolerance condition, obesity,  
10 or a propensity thereto.

18. A method of diagnosing longevity in a patient, said method comprising analyzing the level of PTEN expression or activity in a sample isolated from said patient, whereby a decrease in said level of PTEN expression or activity relative to a control sample is an indication of decreased longevity.

15           19. A method of ameliorating or delaying the onset of an impaired glucose tolerance condition in a patient, said method comprising administering to said patient a therapeutically-effective amount of a compound that decreases PTEN expression or activity.

20           20. A method of increasing longevity in a patient, said method comprising administering to said patient a therapeutically-effective amount of

PTEN polypeptide or a compound that increases PTEN expression or activity.

21. The method of claim 19 or 20, wherein said PTEN is human PTEN.

22. A transgenic non-human animal whose cells contain a transgene encoding a mammalian PTEN polypeptide.

5                    23. The transgenic animal of claim 22, wherein said animal is a nematode.

24. The transgenic animal of claim 23, wherein said animal carries a mutation in a *daf-18* gene.

THERAPEUTIC AND DIAGNOSTIC TOOLS  
FOR IMPAIRED GLUCOSE TOLERANCE CONDITIONS

Abstract of the Disclosure

Disclosed herein are novel genes and methods for the screening of  
5 therapeutics useful for treating impaired glucose tolerance conditions, as well as  
diagnostics and therapeutic compositions for identifying or treating such  
conditions.

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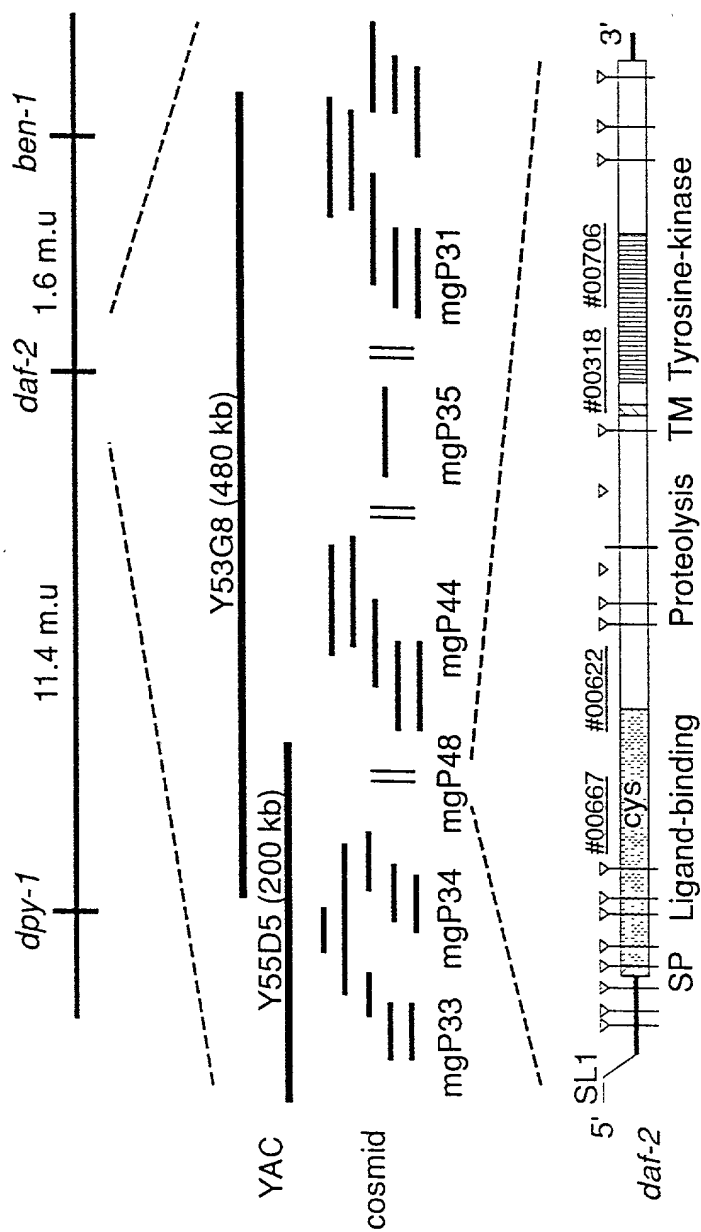


Fig. 1

1 MTSLMLLLLFAFVQPCASIVEKRCGPIDIRNRPWDIKPQWSKLGDPNEKDLAQRMVNCT  
 61 VVEGSLTISFVLKHKTKAQEEMHRSLOPRYSQDEFITFPHLREITGTLLVFETEGLVDLR  
 121 KIFPNLRVIGGRSLIQHYALIIYRNPDL EIGLDKLSVIRNGGVRIIDNRKLCYTKTIDWK  
 181 HLITSSINDVVVDNAAEYAVTETGLMCPRGACEEDKGESKCHYLEEKNOEQGVERVQSCW  
 241 SNTTCQKSCAYDRLLPTKEIGPGCDANGDRCHDQCVGGCERVNDATAHACKNVYHKGKC  
 301 IEKCDAHLYLLLQORRCVTREOCLQLNPVLSNKTVP IKATAGLCSDKCPDGYQINPDDHRE  
 361 CRKCVGKCEIVCEINHVIDTFPKAQAIRLCNIIDGNLTIEIRGKQDSGMASELKDIFANI  
 421 HTITGYLLVRQSSPFISLNMFRNLRRIEAKSLFRNLYAITVFENPNLKKLFDSTDTLTD  
 481 RGTVSIANNKMLCFKYIKQLMSKLNIPLDPIDQSEGTNGEKAICEDMAINVSITAVNADS  
 541 VFFSWPSFNITDIDQRKFLGYELFFKEVPRIDENMTIEEDRSACVDSWQSVFKQYYETSN  
 601 GEPTPDIFMDIGPRERIRPNTLYAYYVATQMV LHAGAKNGVSKIGFVRTSYYPDPPTLA  
 661 LAQVDSDAIHITWEAPLQPNGDLTHYTIMWRENEVSPYEEAEKFCTDASTPANRQRTKDP  
 721 KETIVADKPVDIPSSRTVAPTLLTMMGHEDQOKTCAATPGCCSCSAIEESSEQNKKRPD  
 781 PMSAIESSAFENKLLDEVLMPRDTMRVRRSIEDANRVSEELEKAENLGKAPKTLGGKKPL  
 841 IHISKKKPSSSSTTSTPAPTIASMYALTRKPTTVPGRIRLYEIEPLPGSWAINVSALA  
 901 LDNSYVIRNLKHYTLYAISLSACQNMTVPGASCSISHRAGALKRTKHITDIDKVLNETIE  
 961 WRFMNN SQQVNTWDPPTFVNGGIFGYVVKLSKVDGSIVMTRCVGAKRGYSTRNQGVLF  
 1021 QNLADGRYFVSVTATSVHGAGPEAESSDPIVVMTPGFFFTVEIILGMLLVFLILMSIAGCI  
 1081 IYYYIQVRYGKKVKALSDFMQLNPEQCVDNKYNADDWELRQDDVVLGQQCGEGSFGKVYL  
 1141 GTGNNVVS LMGDRFGPCA IKINVDDPASTENLNYLMEANIMKNFKTNFIVQLYGVISTVQ  
 1201 PAMVVMEMMDLGNLRDYLRSKREDEVFNETDCNFFDIIPRDKFHEWAAQICDGMAYLESL  
 1261 KFCHRD LAARNCMINRDET VKIGDFGMARDLFQHDQKPSGKRMMFPVRWMSPESLKD GKF  
 1321 DSKSDVWSFGVVL YEMVTLGAQPYIGLSNDEV LNYIGMARKVIKKPECCENYWKVMKMC  
 1381 WRYSRDRPTFLQLVHLIAAEASPEFRDLSFVLTDNQMILDDSEALDLDDIDDDTDMNDQV  
 1441 VEVAPDVENVEVQSDSERRNTDSIPLKQFKTIPPINATTSHSTISIDETPMKAKQREGSL  
 1501 DEEYALMNHSGGPSDAEVRTYAGDGDVERDVRENDVPTRRNTGASTSSYTGGGPYCLTN  
 1561 RGGSNERGAGFGEAVRLTDGVGSGHLNDDDDVEKEISSMDTRRSTGASSSSYGVPTNWS  
 1621 GNRGATYYTSKAQQAATAAAAAAALQQQONGGRGDRLTQLPGTGHLQSTRGGQDGDQIE  
 1681 TEPKNYRNNGSPSRNGNSRDIFNGRSAFGENEHLIEDNEHHPV

Fig. 2A

1	ggtttaatta	cccaagtttg	agctccaaga	gcacacatct	gatcgtcggg
51	ttctactgta	ctccccgaaa	aaccaacaaa	aaacacaagt	ttttgaacac
101	ttgtaaatac	agacagaacg	atgacgagaa	tgaatattgt	cagatgtcgg
151	agacgacaca	aaattttgga	aaattttgga	gaagagaatc	tcggcccagg
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Fig. 2B (sheet 1 of 3)

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Fig. 2B (sheet 2 of 3)

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Fig. 2B (sheet 3 of 3)

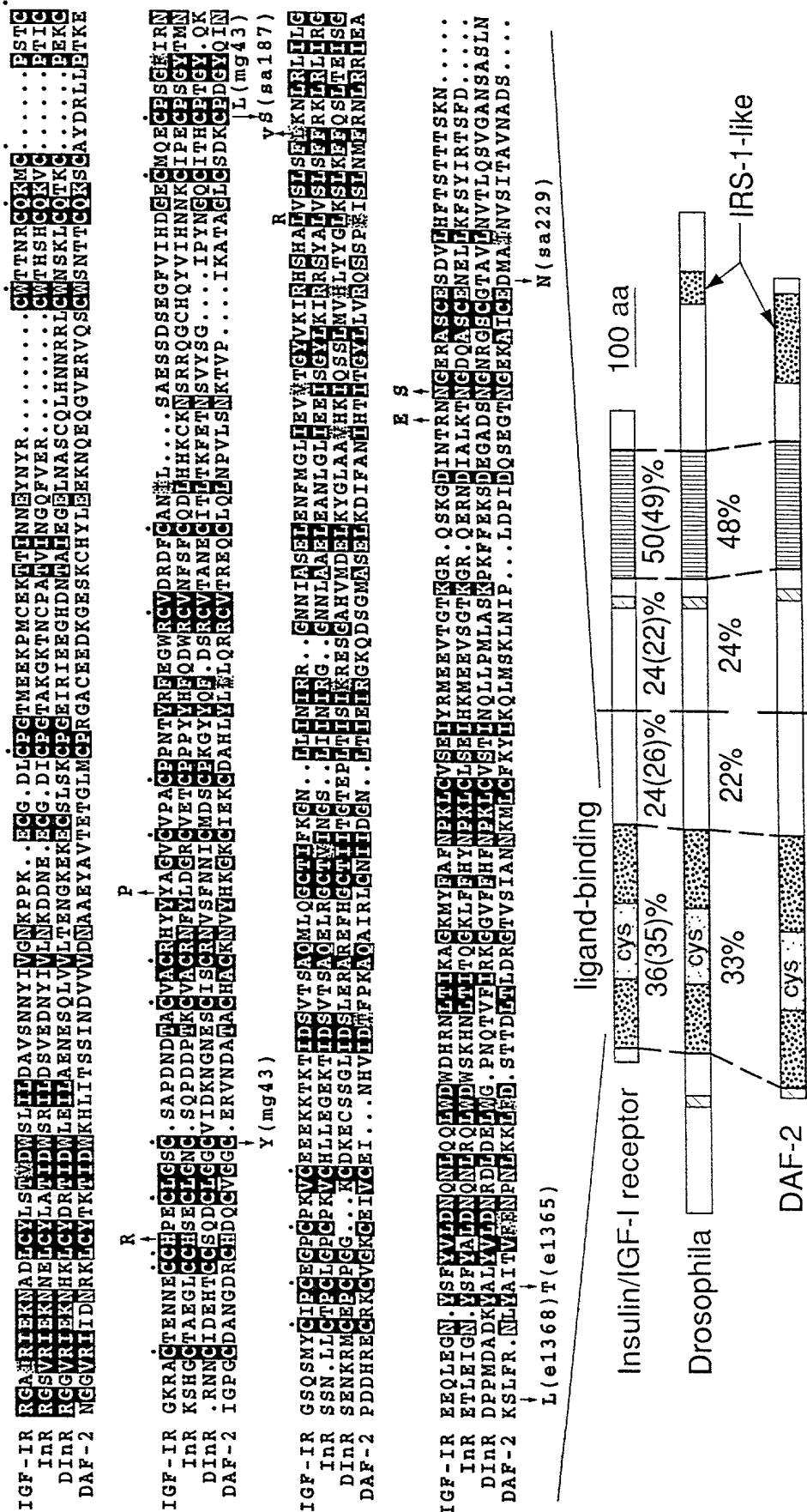
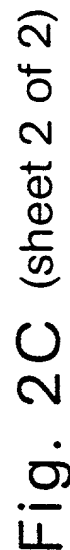


Fig. 2C (sheet 1 of 2)



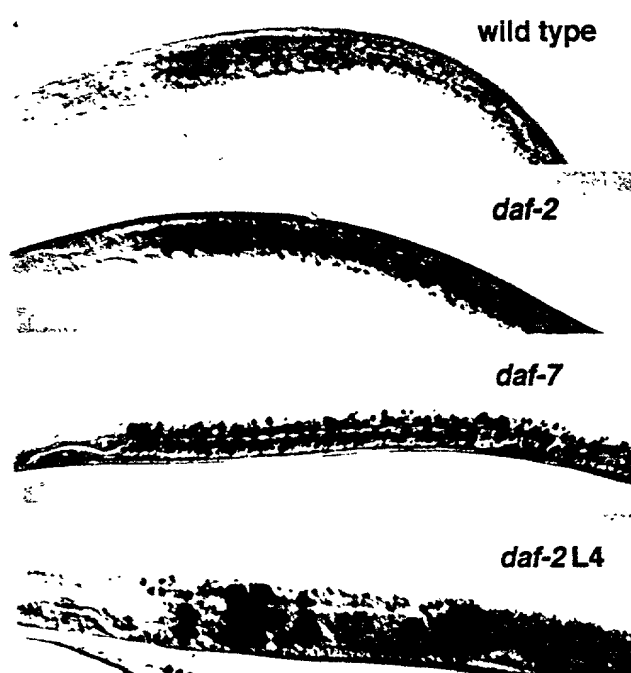


Fig. 3

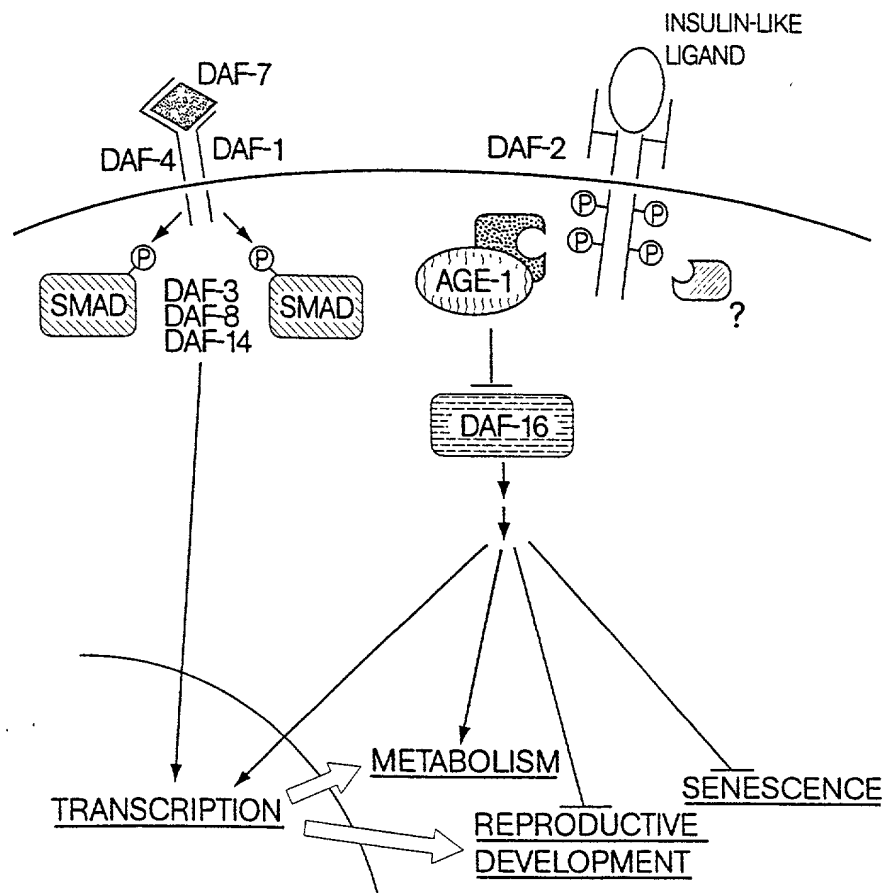


Fig. 4

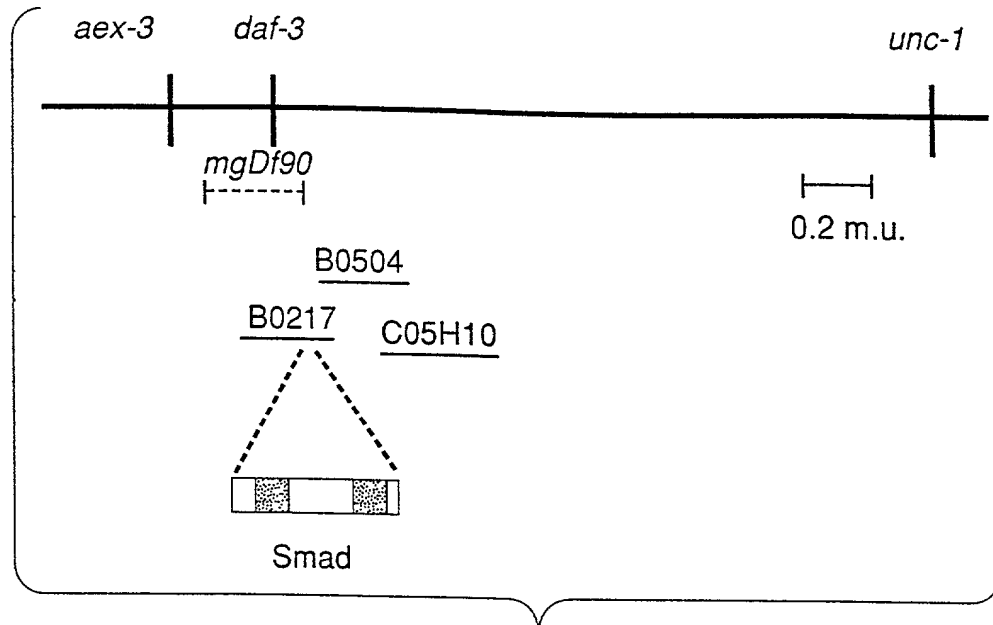


Fig. 5A

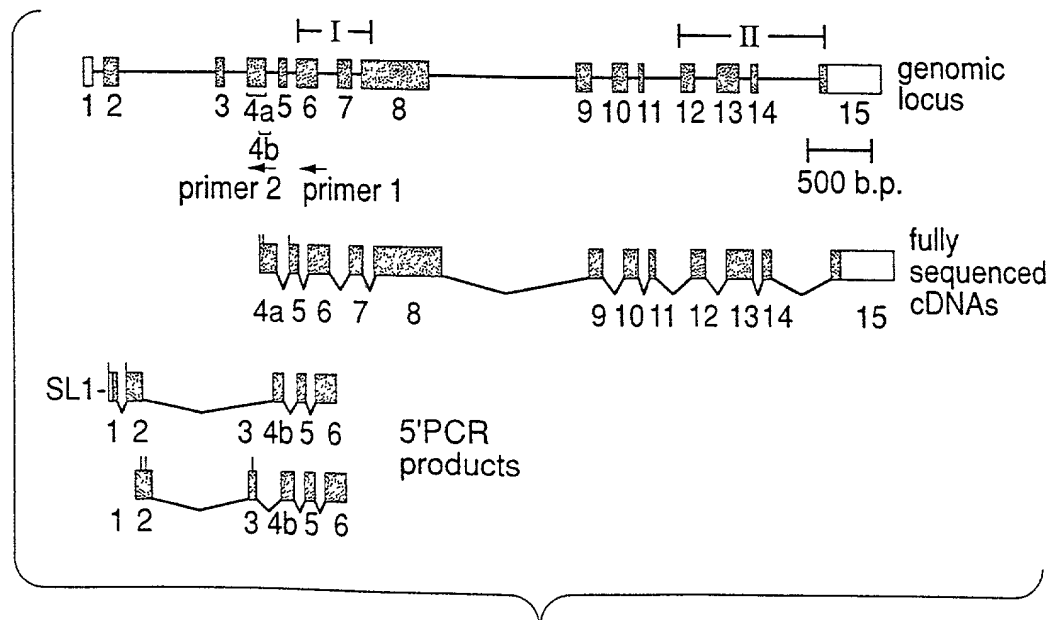


Fig. 5B

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*mg125 P->L*  
RLQVHGRKGFPFHVVYVKLRFRNEMTKNETRHVDHCKHAFEMKSDMVCVNPHY  
| | | | | | | | | | | | | | | |  
RLQVAGRKGFPFHV IYARLWRWPD LHKNELKHVKYCOYAFDLKCDSVCVNPHY

DAF-3 IVVYEEKNLQIGE..KKCSRGNFHVDGGFI..CSENRYSLGLEPNPIREPVAFKV  
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DPC4 IAYFEMDVQVGGETFKVPSSCPIVTVDGYVDPSSGDRFCGLQLSNVHRTEAIERA  
*mg132* G->E  
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| | | | | | | | | | | | | | | | | |  
RLHIGKGVQLECKGEADVWRCLSDHAVFVQSYLLDREAGRAPGDAVHKIYPSA  
  
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| | | | | | | | | | | | | | | | | |  
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Fig. 5C

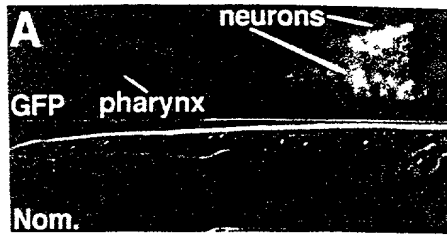


Fig. 6A

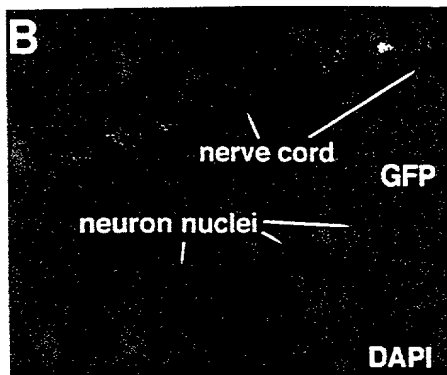


Fig. 6B

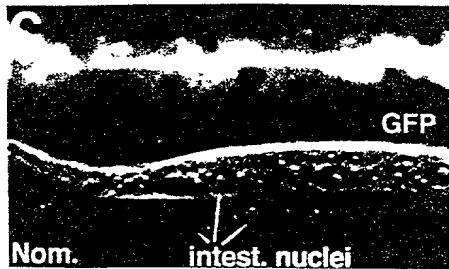


Fig. 6C

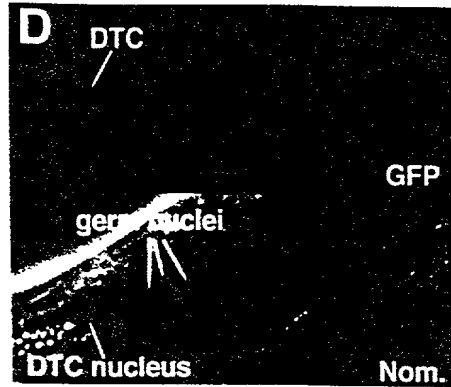


Fig. 6D



Fig. 6E

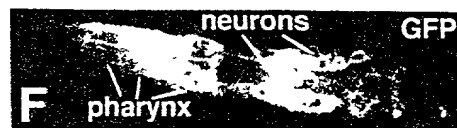


Fig. 6F



Fig. 6G

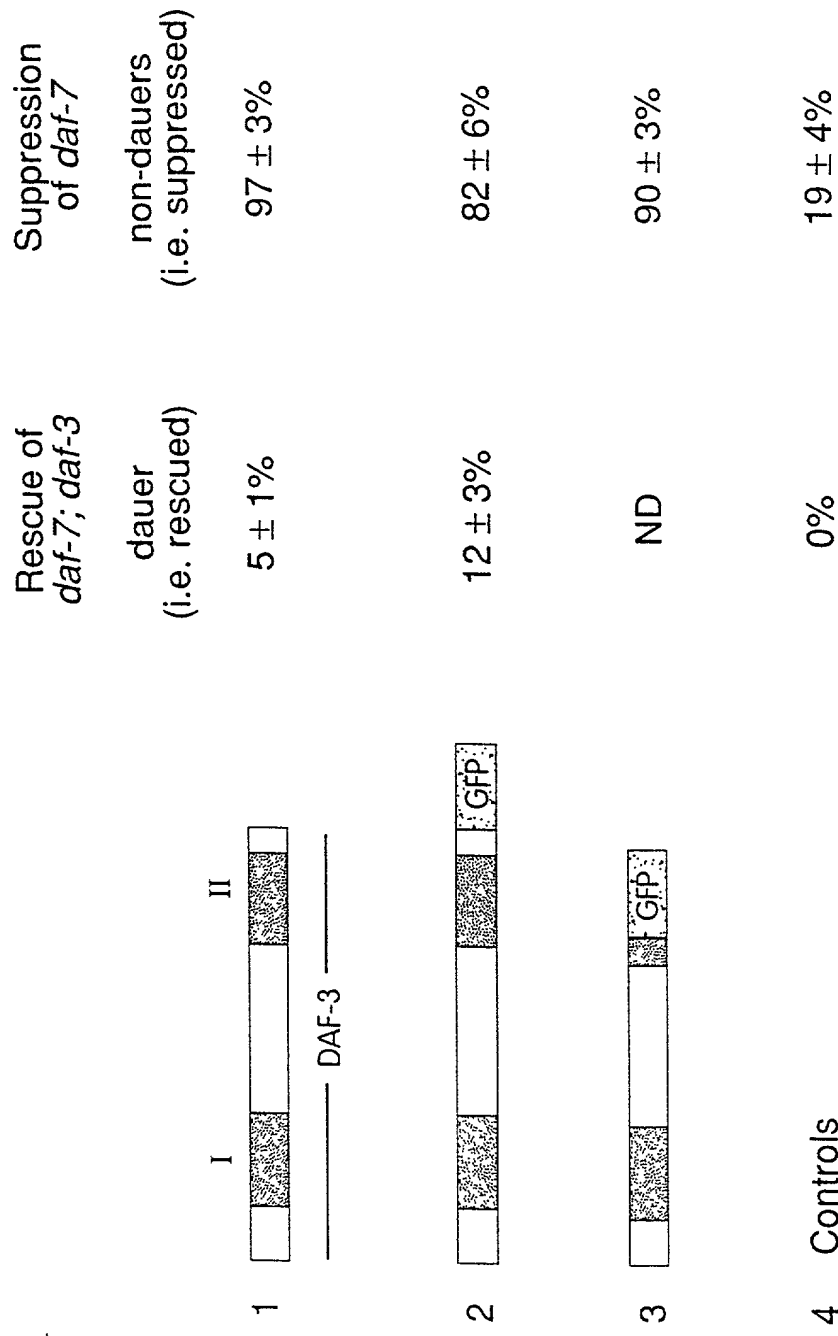


Fig. 7

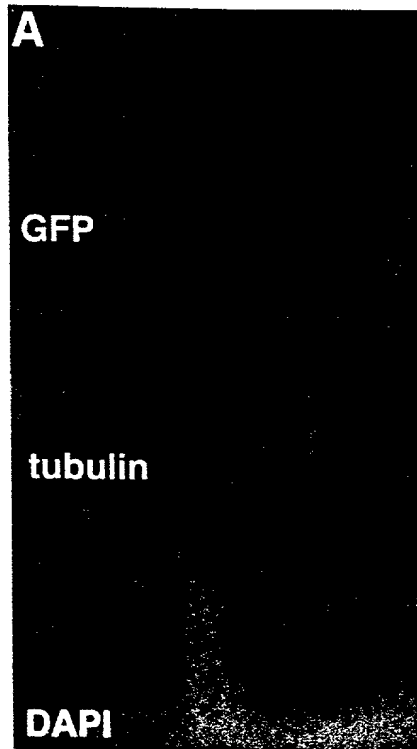


Fig. 8A

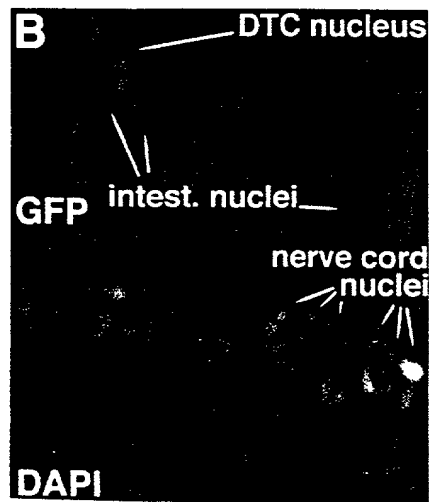


Fig. 8B

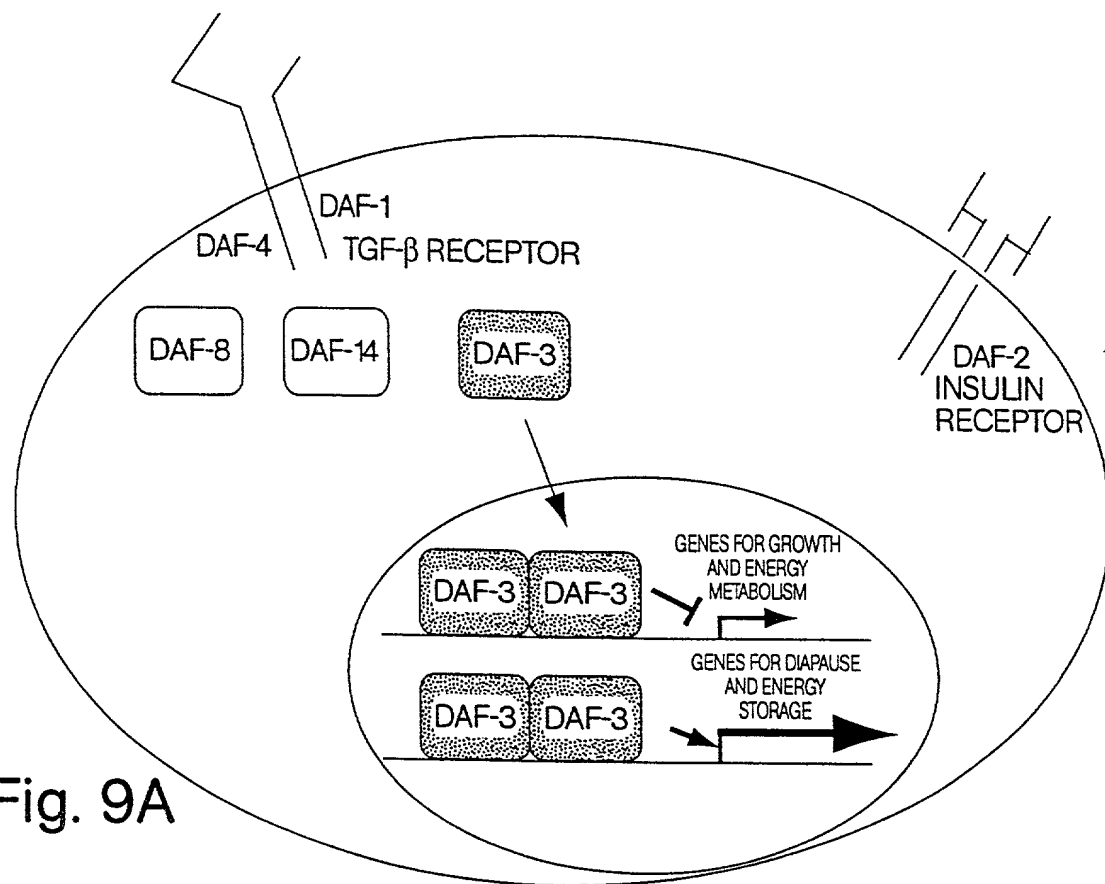


Fig. 9A

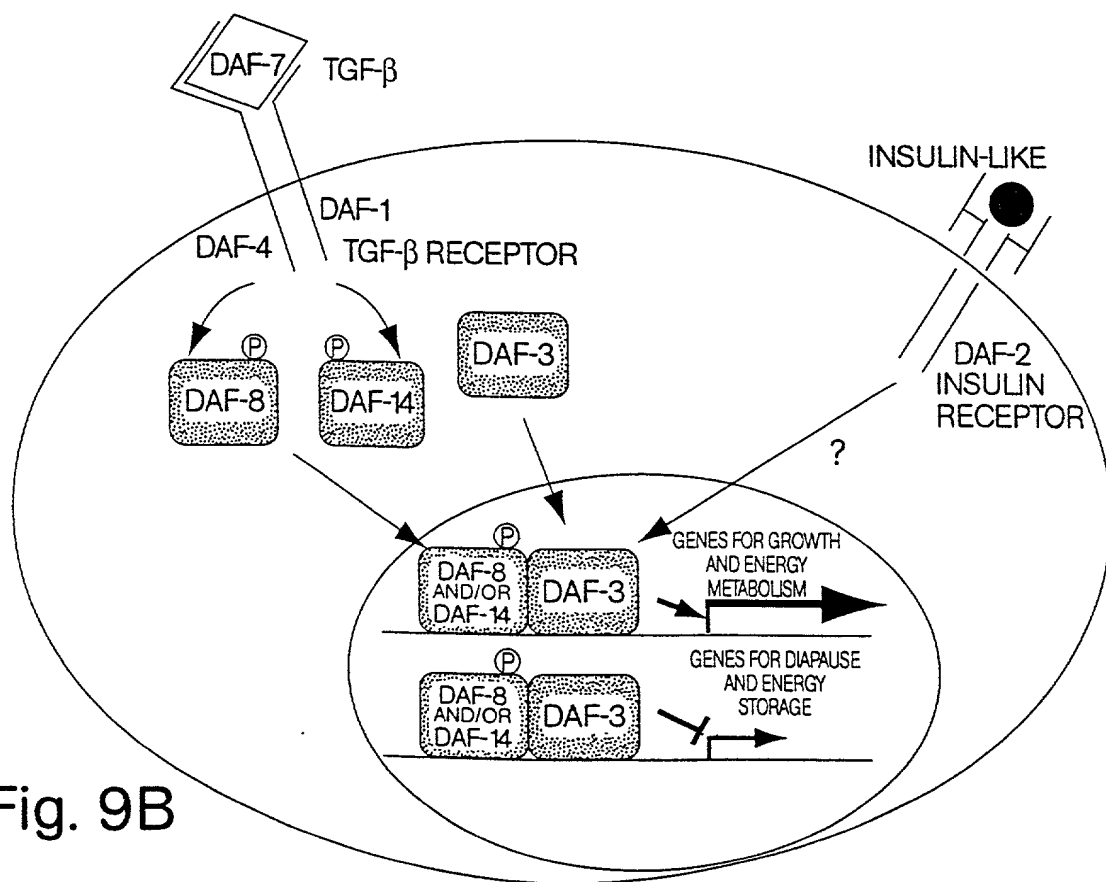


Fig. 9B

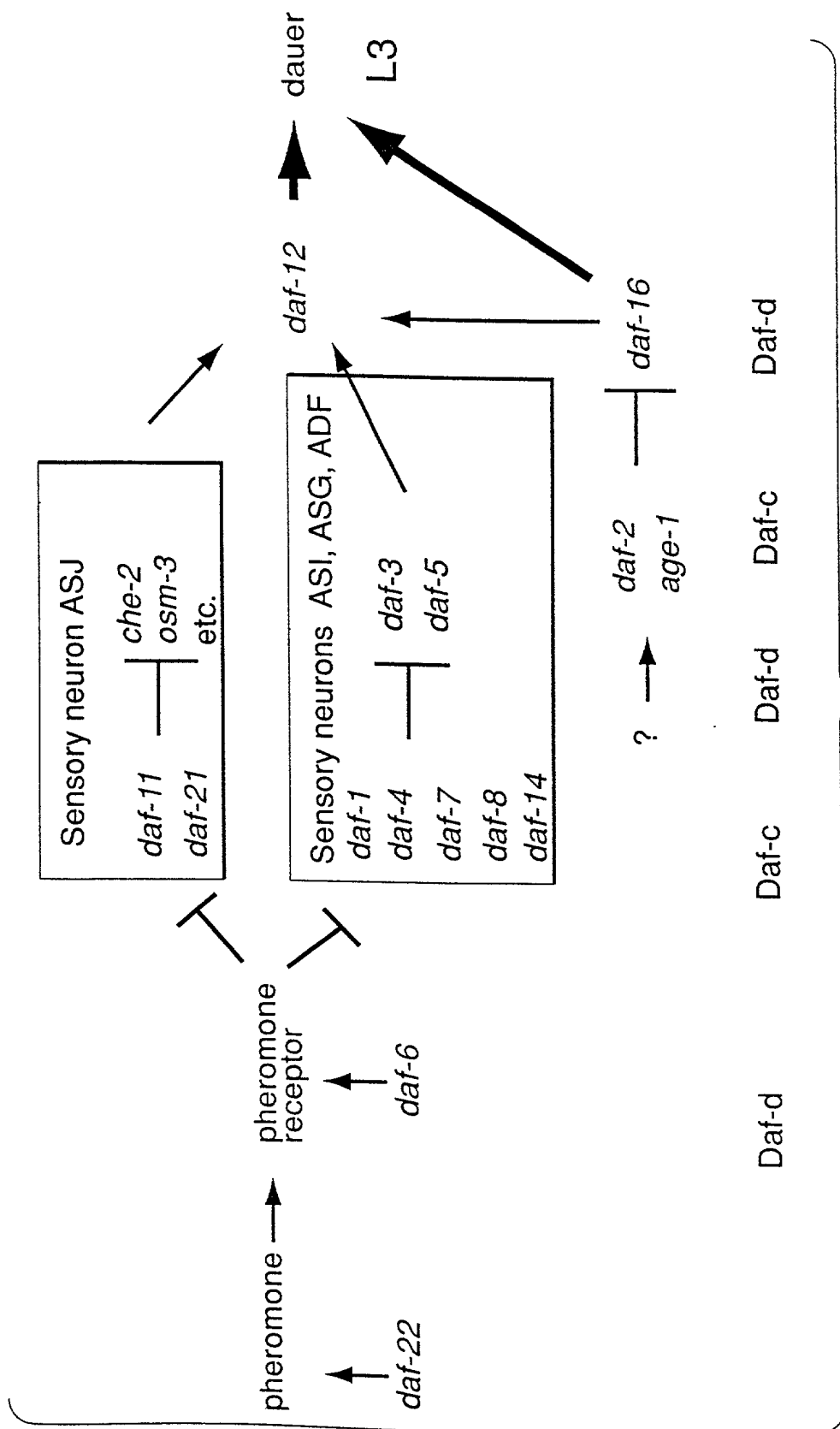


Fig. 10

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 201 aaatatgacc gagtacgatt tggatgtgtt gaagcttgga aaaccagcag  
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Fig. 11A (sheet 1 of 2)

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Fig. 11A (sheet 2 of 2)

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 701 gaaggataag aagaatgatc tccagaacct gattgatgtg gttctttcaa  
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 801 cggttacagg tccacggaag aaaaggtttc cctcacgtag tctatggcaa  
 851 actgtggagg tttaatgaaa tgacaaaaaa cgaaacgcgt catgtggacc  
 901 actgcaagca cgcatttgaa atgaaaagt acatgggatg cgtgaatccc  
 951 tatcactacg aaattgtcat tggaactatg attgttgggc agagggatca  
 1001 tgacaatcga gatatgccgc cgccacatca acgctaccac actccaggtc  
 1051 ggcaggatcc agttgacgat atgagtagat ttataccacc agcttccatt  
 1101 cgtccgcctc cgatgaacat gcacacaagg cctcagccta tgcctcaaca  
 1151 attgccttca gttggcgcaa cgtttgcccc tcctctccca catcaggcgc  
 1201 cacataacc aggggtttca catccgtact ccattgctcc acagacccat  
 1251 taccggttga acatgaaccc aattccgcaa atgccgcaa tgccacaaat  
 1301 gccaccacct ctccatcagg gatattggaat gaatgggccc agttgctctt  
 1351 cagaaaacaa caatccattc caccaaaatc accattataa tgatattagc  
 1401 catccaaatc actattccta cgactgtggt ccgaacttgt acgggtttcc  
 1451 aactccttat ccggattttc accatccttt caatcagcaa ccacaccagc  
 1501 cgccacaact atcacaaaac catacgtccc aacaaggcag tcatcaacca  
 1551 gggcaccaag gtcagggtacc gaatgatcca ccaatttcaa gaccagtgtt  
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 1651 catttggaat tcgatttttt gaaggagaaa gtgaacaatc cggcgcaata  
 1701 attcgggtcta gtaacaaatt cattgaagaa tttgattcgc cgatttgtgg  
 1751 tgtgacagtt gttcgaccgc ggatgacaga cggtagggtt ttggagaaca  
 1801 tcatgccgga agatgcacca tatcatgaca tttgcaagtt cattttgagg  
 1851 ctacacatcag aaagtgtaac tttctcagga gaggggcccag aagttagtga  
 1901 tttgaacgaa aaatggggaa caattgtgta ctatgagaaa aatttgcaaa  
 1951 ttggcgagaa aaaatgttcg agaggaaatt tccacgtgga tggcggattc  
 2001 atttgctctg agaatcggtta cagtctcgga cttgagccaa atccaattag  
 2051 agaaccagtg gcgtttaaag ttcgtaaagc aatagtggat ggaattcgct

Fig. 11B (sheet 1 of 2)

2101 tttcctacaa aaaagacggg agtgtttggc ttcaaaaccg catgaagtac  
 2151 ccggtatttg tcacttctgg gtatctcgac gagcaatcag gaggcctaaa  
 2201 gaaggataaa gtgcacaaag tttacggatg tgcgtctatc aaaacgtttg  
 2251 gcttcaacgt ttccaaacaa atcatcagag acgcgcttct ttccaagcaa  
 2301 atggcaacaa tgtacttgca aggaaaattg actccgatga attatatcta  
 2351 cgagaagaag actcaggaag agctgcgaag ggaagcaaca cgcaccactg  
 2401 attcattggc caagtactgt tgtgtccgtg tctcgttctg caaaggattt  
 2451 ggagaagcat acccagaacg cccgtcaatt catgattgtc cagtttggat  
 2501 tgagttgaaa atcaacattg cctacgattt catggattca atctgccagt  
 2551 acataacc aa ctgcttcgag ccgctaggaa tggaagattt tgcaaaattg  
 2601 ggaatcaacg tcagtgatga ctaa atgata acttttttca ctcaccctac  
 2651 tagatactga tttagtctta ttccaaatca tccaacgata tcaaactttt  
 2701 tcctttgaac tttgcatact atgttatcac aagttccaag cagtttcaat  
 2751 acaaacatag gatatgttaa caacttttga taagaatcaa gttaccaact  
 2801 gttcattgtg agctttgagc tgtatagaag gacaatgtat ccataacctc  
 2851 aatctttaat agtcatcagt cactggtccc gcaccaattt tttcgattcg  
 2901 catatgtcat atattgcacc gtggcccttt ttattgtaac ttttaatata  
 2951 ttttcttccc aacttgtgaa tatgattgat gaaccaccat tttgagtaat  
 3001 aaatgtattt tttgtgg

Fig. 11B (sheet 2 of 2)

1 gtaatcaaat tgtaaaggaa aaatattaat agtcagagta cacataaatg  
 51 ggtgatcatc ataatttaac gggccttccc ggtacctcca tcccgccaca  
 101 gttcaactat tctcagcccc gtaccagcac cggaggcccc ctttatggtg  
 151 gaaaaccttc tcatggattg gaagatattc ctgatgtaga ggaatatgag  
 201 aggaacctgc tcggggctgg agcagggttt aatctgctca atgtaggaaa  
 251 tatggctaata gaatttaaac caataatcac attggacacg aaaccacctc  
 301 gtgatgccaa caagtcattg gcattcaatg gcgggttgaa gctaatactc  
 351 ccgaaaactg aagttcccg aagcacacac ccgatgatgt caccagtga  
 401 tacaactaca aagattctac aacggagtgg tattaataatg gaaatccccg  
 451 catatttgga tccagacagt caggatgatg acccggaaga tgggtgtcaac  
 501 tacccggtac cagatttatt tgacacaaaa aacacaaata tgaccgagta  
 551 cgatttggtat gtgttgaaagc ttggaaaacc agcagtagat gaagcacgga  
 601 aaaagatcga agttcccgac gctagtgcgc cgccaaacaa aattgtagaa  
 651 tatttgatgt attatagaac gttaaaaagaa agtgaactca tacaactgaa  
 701 tgcgtatcgg acaaaacgaa atcgattatc gttgaacttg gtcaaaaaaca  
 751 atattgatcg agagttcgac caaaaagctt gcgagtcctt ggtgaaaaaaa  
 801 ttgaaggata agaagaatga tctccagaac ctgattgatg tgggtctcttc  
 851 aaaaggtaca aaatataccg gttgcattac aattccaagg acacttgatg  
 901 gccgggtaca ggtccacgga agaaaagggt tccctcacgt agtctatggc  
 951 aaactgtgga ggtttaatga aatgacaaaa aacgaaacgc gtcattgtgga  
 1001 ccactgcaag cagcatttg aaatgaaaag tgacatggta tgcgtgaatc  
 1051 cctatcacta cgaaattgtc attggaacta tgattgttg gtagaggat  
 1101 catgacaatc gagatatgcc gccgccacat caacgctacc acactccagg  
 1151 tcggcaggat ccagttgacg atatgagtag atttatacca ccagcttcca  
 1201 ttcgtccgcc tccgatgaac atgcacacaa ggcctcagcc tatgcctcaa  
 1251 caattgcctt cagttggcgc aacgtttgcc catcctctcc cacatcaggc  
 1301 gccacataac ccagggggtt cacatccgta ctccattgct ccacagacc  
 1351 attaccggtt gaacatgaac ccaattccgc aaatgccgca aatgccacaa  
 1401 atgccaccac ctctccatca gggatatgga atgaatgggc cgagttgctc  
 1451 ttcagaaaaa aacaatccat tccacaaaaa tcaccattat aatgatatta  
 1501 gccatccaaa tcaactattc tacgactgtg gtccgaactt gtacgggttt  
 1551 ccaactcctt atccggattt tcaccatcct ttcaatcagc aaccacacca  
 1601 gccgccacaa ctatcacaaa accatacgtc ccaacaaggc agtcatcaac  
 1651 cagggcacca aggtcaggta ccgaatgatc caccaatttc aagaccagt  
 1701 ttacaaccat caacagtcac cttggacgtg ttccgtcggg actgtagaca  
 1751 gacatttgga aatcgatttt ttgaaggaga aagtgaacaa tccggcgcaa  
 1801 taattcgggtc tagtaacaaa ttcattgaag aatttgattc gccgatttgt  
 1851 ggtgtgacag ttgttcgacc gcggatgaca gacggtgagg ttttgagaa  
 1901 catcatgccg gaagatgcac catatcatga catttgcaag ttcattttga  
 1951 ggctcacatc agaaagtgt actttctcag gagaggggcc agaagttagt  
 2001 gatttgaacg aaaaatgggg aacaattgtg tactatgaga aaaatttgca  
 2051 aattggcgag aaaaaatgtt cgagaggaaa tttccacgtg gatggcggat

Fig. 11C (sheet 1 of 2)

2101 tcatttgctc tgagaatcgt tacagtctcg gacttgagcc aaatccaatt  
 2151 agagaaccag tggcgtttaa agttcgtaaa gcaatagtgg atggaattcg  
 2201 cttttcctac aaaaaagacg ggagtgtttg gcttcaaaac cgcatagaat  
 2251 acccggattt tgtcacttct gggatatctcg acgagcaatc aggaggccta  
 2301 aagaaggata aagtgcacaa agttttacgga tgtgcgtcta tcaaaacgtt  
 2351 tggcttcaac gtttccaaac aaatcatcag agacgcgctt ctttccaagc  
 2401 aaatggcaac aatgtacttg caaggaaaat tgactccgat gaattatatc  
 2451 tacgagaaga agactcagga agagctgcga aggaagcaa cacgcaccac  
 2501 tgattcattg gccaaagtact gttgtgtccg tgtctcgttc tgcaaaggat  
 2551 ttggagaagc ataccagaa cgcccgtcaa ttcatagattg tccagtttgg  
 2601 attgagttga aaatcaacat tgcctacgat ttcataggatt caatctgcca  
 2651 gtacataacc aactgcttcg agccgctagg aatggaagat ttgcaaaat  
 2701 tgggaatcaa cgtcagtgat gactaaatga taactttttt cactcaccct  
 2751 actagatact gatttagtct tattccaaat catccaacga tatcaaactt  
 2801 tttcctttga actttgcata ctatgttatc acaagttcca agcagtttca  
 2851 atacaaacat aggatatgtt aacaactttt gataagaatc aagttaccaa  
 2901 ctggttcattg tgagctttga gctgtataga aggacaatgt atcccatacc  
 2951 tcaatcttta atagtcatca gtcactggtc ccgcaccaat tttttcgatt  
 3001 cgcataatgtc atatattgca ccgtggccct ttttattgta actttttaata  
 3051 tattttcttc ccaacttggtg aatatgattg atgaaccacc attttgagta  
 3101 ataaatgtat tttttgtgg

Fig. 11C (sheet 2 of 2)

1 MKLIATSLLV PDEHTPMMSP VNTTTKILQR SGIKMEIPPY LDPDSQDDDP  
 51 EDGVNYPDPD LFDTKNTNMT EYDL DV LKLG KPAVDEARKK IEVPDASAPP  
 101 NKIVEYLMYY RTLKESELIQ LNAYRTKRNR LSLNLVKNNI DREFDQKACE  
 151 SLVKKLKDKK NDLQNLIDVV LSKGTKYTGC ITIPRTL DGR LQVHGRKGFP  
 201 HVVYGKLWRF NEMTKNETRH VD HCKHAFEM KSDMVCVNPY HYEIVIGTMI  
 251 VGQRDHDNRD MPPPHQRYHT PGRQDPVDDM SRFIPPASIR PPPMNMHTRP  
 301 QPMPQQLPSV GATFAHPLPH QAPHNPGVSH PYSIAPQTHY PLN MNPIPQM  
 351 PQMPQMPPPL HQGYGMNGPS CSSEN NNPFH QNH HYNDISH PNHYSYDCGP  
 401 NLYGFPTYP DFHHPFNQQP HQPPQLSQNH TSQQGSHQPG HQGQVPNDPP  
 451 ISRPVLQPST VTLDVFRRYC RQTFGNRFFE GESEQSGAII RSSNKFIEEF  
 501 DSPICGVTVV RPRMTDGEVL ENIMPEDAPY HDICKFILRL TSESVTFSGE  
 551 GPEVSDLNEK WGTIVYYEKN LQIGEKKCSR GN FHV DGGFI CSENRYSLGL  
 601 EPNPIREPVA FKVRKAIVDG IRFSYKKDGS VWLQNRMKYP VFV TSGYLDE  
 651 QSGGLKKDKV HKVYGCASIK TFGFNVSKQI IRDALLSKQM ATMYLQGKLT  
 701 PMNYIYEKKT QEELRREATR TTDSLAKYCC VRVSFCKGFG EAYPERPSIH  
 751 DCPVWIELKI NIAYDFMDSI CQYITNCFEP LGMEDFAKLG INVSD

Fig. 12A

1 MGDHNLTLGL PGTSIPPQFN YSQPGTSTGG PLYGGKPSHG LEDIPDVEEY  
51 ERNLLGAGAG FNLLNVGNMA NVPDEHTPMM SPVNTTTKIL QRSIKMEIP  
101 PYLDPDSQDD DPEDGVNYPD PDLFDTKNTN MTEYDLVLK LGKPAVDEAR  
151 KKIEVPDASA PPNKIVEYLM YYRTLKESEL IQLNAYRTKR NRLSLNLVKN  
201 NIDREFDQKA CESLVKKLKD KKNDLQNLID VVLSKGTKYT GCITIPRTLD  
251 GRLQVHGRKG FPHVVYGLW RFNEMTKNET RHVDHCKHAF EMKSDMVCVN  
301 PYHYEIVIGT MIVGQRDHDN RDMPPPHQRY HTPGRQDPVD DMSRFIPPAS  
351 IRPPPMNMHT RPQPMPQQLP SVGATFAHPL PHQAPHNPGV SHPYSIAPQT  
401 HYPLNMNPIP QMPQMPQMPP PLHQGYGMNG PSCSSENNNP FHQNHXYNDI  
451 SHPNHYSYDC GPNLYGFPTP YPDFHHFNFQ QPHQPPQLSQ NHTSQQGS HQ  
501 PGHQGVVPND PPISRPVLQP STVTLDVFR YCRQTFGNRF FEGESEQSGA  
551 IIRSSNKFIE EFDSPICGVT VVRPRMTDGE VLENIMPEDA PYHDICKFIL  
601 RLTSSESVTFS GEGPEVSDLN EKWGTIVYYE KNLQIGEKKC SRGNFHV DGG  
651 FICSENRYSL GLEPNPIREP VAFKVRKAIV DGIRFSYKKD GSVWLQNRMK  
701 YPVFVTSGYL DEQSGGLK KD KVHKVYGCAS IKTFGFNVSK QIIRDALLSK  
751 QMATMYLQ GK LTPMNYIYEK KTQEELRREA TRTDSLAKY CCVRVSFCKG  
801 FGEAYPERPS IHDCPVWIEL KINIAYDFMD SICQYITNCF EPLGMEDFAK  
851 LGINVSDD

Fig. 12B

1 MGDHHNLTGL PGTSIPPQFN YSQPGTSTGG PLYGGKPSHG LEDIPDVEEY  
 51 ERNLLGAGAG FNLLNVGNMA NEFKPIITLD TKPPRDANKS LAFNGGLKLI  
 101 TPKTEVPDEH TPMMSPVNTT TKILQRSGIK MEIPPYLDPD SQDDDPEDGV  
 151 NYPDPDLFDT KNTNMTEYDL DVLKLGKPAV DEARKKIEVP DASAPPNKIV  
 201 EYLMYYRTLK ESELIQLNAY RTKRNRLSLN LVKNNIDREF DQKACESLVK  
 251 KLDKKNLQ NLIDVVLKSG TKYTGCITIP RTLDGRLQVH GRKGFPHVVY  
 301 GKLWRFNEMT KNETRHVDHC KHAFEMKSDM VCVNPHYEI VIGTMIVGQR  
 351 DHDNRDMPPP HQRYHTPGRQ DPVDDMSRFI PPASIRPPPM NMHTRPQMP  
 401 QQLPSVGATF AHPLPHQAPH NPGVSHPYSI APQTHYPLNM NPQPMPQMP  
 451 QMPPPLHQGY GMNGPSCSSE NNNPFHQNH YNDISHPNHY SYDCGPNLYG  
 501 FPTPYPDFHH PFNQQPHQPP QLSQNHTSQG GSHQPGHQGQ VPNDPPISRP  
 551 VLQPSTVTLD VFRRYCRQTF GNRFFEGESE QSGAIRSSN KFIEEFDSP  
 601 CGVTVVRPRM TDGEVLENIM PEDAPYHDIC KFILRLTSES VTFSGEGPEV  
 651 SDLNEKWGTI VYIEKNLQIG EKKCSRGNFH VDGGFICSEN RYSLGLEPNP  
 701 IREPVAFKVR KAIVDGIRFS YKKGDSVWLQ NRMKYPVFVT SGYLDEQSGG  
 751 LKKDKVHKVY GCASIKTFGF NVSKQIIRDA LLSKQMATMY LQGLTPMNY  
 801 IYEKKTQEEL RREATRTTDS LAKYCCVRVS FCKGFGEAYP ERPSIHDCPV  
 851 WIELKINIAY DFMD SICQYI TNCFEPLGME DFAKLGINVS DD

Fig. 12C

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 gaatcctccgtatacacacacacatagtaattctacctccaaaattttactgaaagatgtgatccccctctctgtctccctc  
 taaaaacattatttgtctgtttgtgtatattgccaccacgtcgattttaaattaaaaccatcggtttttcttctttct  
 acttttttctcgaaaaatttaacaacacacaaaaaatccttcaaaaaatctcagttttaaatgggtgtggcaatatatcg  
 gatccccctctacaccagaacagtccttgcaatttcagagaatgattttcagatttttcatatcacaggccccctttttt  
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 tcgtctccctccgcccccaatatatttgcgactgtatgatgatgatgatttaataaaaaat

Fig. 13B

MMEMLVDQGTDASSASTSTSSVSFRGADTFMNTPDVMMNDMEPIPRDR  
 CNTWPMRRPQLEPPLNSSPIIHEQIPEEDADLYGSNEQCGQLGGASSNGST  
 AMLHTPDGNSHQTSPSDFRMSSEPDFTVSGKKTTRRNAWGNMSYAEI  
 TTAIMASPEKRLTLAQVYEWVQNVFYFRDKGDSNSSAGWKNSIRHNLSLH  
 SRFMRIQNEGAGKSSWWVINPDAKPGMNPRTTRERSNTIETTTKAQLEKSR  
 RGAKKRIKERALMGSLHSTLNGNSIAGSIQTISHDLYDDDSMQGAFDNPVS  
 SFRPRTQSNLSIPGSSSRVSPAIGSDIYDDLEFPVSWVGESVPAIPSDIVDR  
 TDQMRIDATTHIGGVQIKQESKPIKTEPIAPPPSYHELNSVRGSCAQNPLL  
 RNPIVPSTNFKPMPLPGAYGNYQNGGITPINWLSTSNSSPLPGIQSCGIVA  
 AQHTVASSSALPIDLENLTLPDQPLMDTMDVDALIRHELSQAGGQHIHFDL

Fig. 14A

MQQYIYQESSATIPHHHLNQHNPNPYHPMHPHHQLPHMQQLPQPLNLNMTT  
 LTSSGSSVASSIGGGAQCSPCASGSSTAATNSSQQQQTQVQMLAASVPCSS  
 SGMTLGMSLNLSSQGGGPMPAKKKRCRKKPTDQLAQKKPNPWGEESYSDIIA  
 KALESAPDGRKLNEIYQWFSDNIPYFGERSSPPEEAAGWKNSIRHNLSLH  
 RFMRIQNEGAGKSSWWVINPDAKPGMNPRTTRERSNTIETTTKAQLEKSR  
 GAKKRIKERALMGSLHSTLNGNSIAGSIQTISHDLYDDDSMQGAFDNPSS  
 FRPRTQSNLSIPGSSSRVSPAIGSDIYDDLEFPVSWVGESVPAIPSDIVDR  
 TDQMRIDATTHIGGVQIKQESKPIKTEPIAPPPSYHELNSVRGSCAQNPLLR  
 NPIVPSTNFKPMPLPGAYGNYQNGGITPINWLSTSNSSPLPGIQSCGIVAA  
 QHTVASSSALPIDLENLTLPDQPLMDTMDVDALIRHELSQAGGQHIHFDL

Fig. 14B

1 cggaagccat ggagctcgag atctgattgc tggacacgga cggaactccg acgtatctcg  
 61 cagatgcatg ttaacatttt acatccacaa ctgcaaacga tggctcgagca gtggcaaagt  
 121 cgagaacgcc catcgctgga gaccgagaat ggcaaaggat cgctgctcct ggaaaatgaa  
 181 ggtgtcgcag atatcatcac tatgtgtcca ttccggagaag ttattagtgt agtatttccg  
 241 tggtttcttg caaatgtgcg aacatcgcta gaaatcaagc tatcagattt caaacatcaa  
 301 cttttcgaat tgattgctcc gatgaagtgg ggaacatatt ccgtaaagcc acaggattat  
 361 gtgttcagac agttgaataa tttcggcgaa attgaagtta tatttaacga cgatcaaccc  
 421 ctgtcgaaat tagagctcca cggcactttc ccaatgcttt ttctctacca acctgatgga  
 481 ataaacaggg ataaagaatt aatgagtgat ataagtcatt gtctaggata ctactggat  
 541 aaactggaag agagcctcga tgaggaactc cgtcaatttc gtgcttctct ctgggctcgt  
 601 acgaagaaaa cgtgcttgac acgtggactt gagggtagca gtcactacgc gttccccgaa  
 661 gaacagtact tgtgtgttgg tgaatcgtgc ccgaaagatt tggaaatcaaa agtcaaggct  
 721 gccagctga gttatcagat gttttggaga aaacgtaaag cggaaatcaa tggagtgtgc  
 781 gagaaaatga tgaagattca aattgaattc aatccgaacg aaactccgaa atctctgctt  
 841 cacacgtttc tctacgaaat gcgaaaattg gatgtatacg ataccgatga tcctgcagat  
 901 gaaggatggt ttcttcaatt ggctggacgt accacgtttg ttacaaatcc agatgtcaaa  
 961 cttacgtctt atgatggtgt ccgttcggaa ctggaaagct atcgatgcc tggattcggt  
 1021 gttcgccgac aatcactagt cctcaaagac tattgtcgcc caaaaccact ctacgaacca  
 1081 cattatgtga gagcacacga acgaaaactt gctctagacg tgctcagcgt gtctatagat  
 1141 agcacaccaa aacagagcaa gaacagtgc atggttatga ctgattttcg tccgacagct  
 1201 tactcaaac aagtttact ttgggacctt gacgcgaatc ttatgatacg gcctgtgaat  
 1261 atttctggat tcgattttcc ggccgacgtg gatatgtacg ttcgaatcga attcagtga  
 1321 tatgtgggga cactgacgct ggcatacaaa tctacaacaa aagtgaatgc tcaatttgca  
 1381 aaatggaata aggaaatgta cacttttgat ctatacatga aggatatgcc accatctgca  
 1441 gtactcagca ttctgtttt gtacggaaaa gtgaaattaa aaagtgaaga attcgaagtt  
 1501 ggttgggtaa atatgtccct aaccgattgg agagatgaac tacgacaagg acaattttta  
 1561 ttccatctgt gggctcctga accgactgcc aatcgtagta ggatcggaga aaatggagca  
 1621 aggataggca ccaacgcagc gggtacaatt gaaatctcaa gttatggtgg tagagttcga  
 1681 atgccgagtc aaggacaata cacatatctc gtcaagcacc gaagtacttg gacggaaact  
 1741 ttgaatatta tgggtgatga ctatgagtcg tgtatcagag atccaggata taagaagctt  
 1801 cagatgcttg tcaagaagca tgaatctgga attgtattag aggaagatga acaacgtcat  
 1861 gtctggatgt ggaggagata cattcaaaag caggagcctg atttgctcat tgtgctctcc  
 1921 gaactcgcat ttgtgtggac tgatcgtgag aacttttccg agctctatgt gatgcttgaa  
 1981 aaatggaaac cgccgagtgt ggcagccgcg ttgactttgc ttggaaaacg ttgcacggat  
 2041 cgtgtgattc gaaagtgtgc agtgagagaag ttgaatgagc agctgagccc ggtcacattc  
 2101 catcttttca tattgcctct catacaggcg ttgaagtacg aaccgcgtgc tcaatcggaa  
 2161 gttggaatga tgctcttgac tagagctctc tgcgattatc gaattggaca tcgacttttc  
 2221 tggctgctcc gtgcagagat tgctcgtttg agagattgtg atctgaaaag tgaagaatat  
 2281 cgccgtatct cacttctgat ggaagcttac ctccgtggaa atgaagagca catcaagatc  
 2341 atcacccgac aagttgacat ggttgatgag ctacacagaa tcagcactct tgtcaaagga  
 2401 atgcaaaaag atgttgctac gatgaaactg cgtgacgagc ttcgatcgat tagtcataaa  
 2461 atggaaaata tggattctcc actggatcct gtgtacaaac tgggtgaaat gataatcgac  
 2521 aaagccatcg tcctaggaag tgcaaaacgt ccgttaatgc ttcactggaa gaacaaaaat  
 2581 ccaaagagtg acctgcacct tccgttctgt gcaatgatct tcaagaatgg agacgatctt  
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Fig. 15 (sheet 1 of 2)

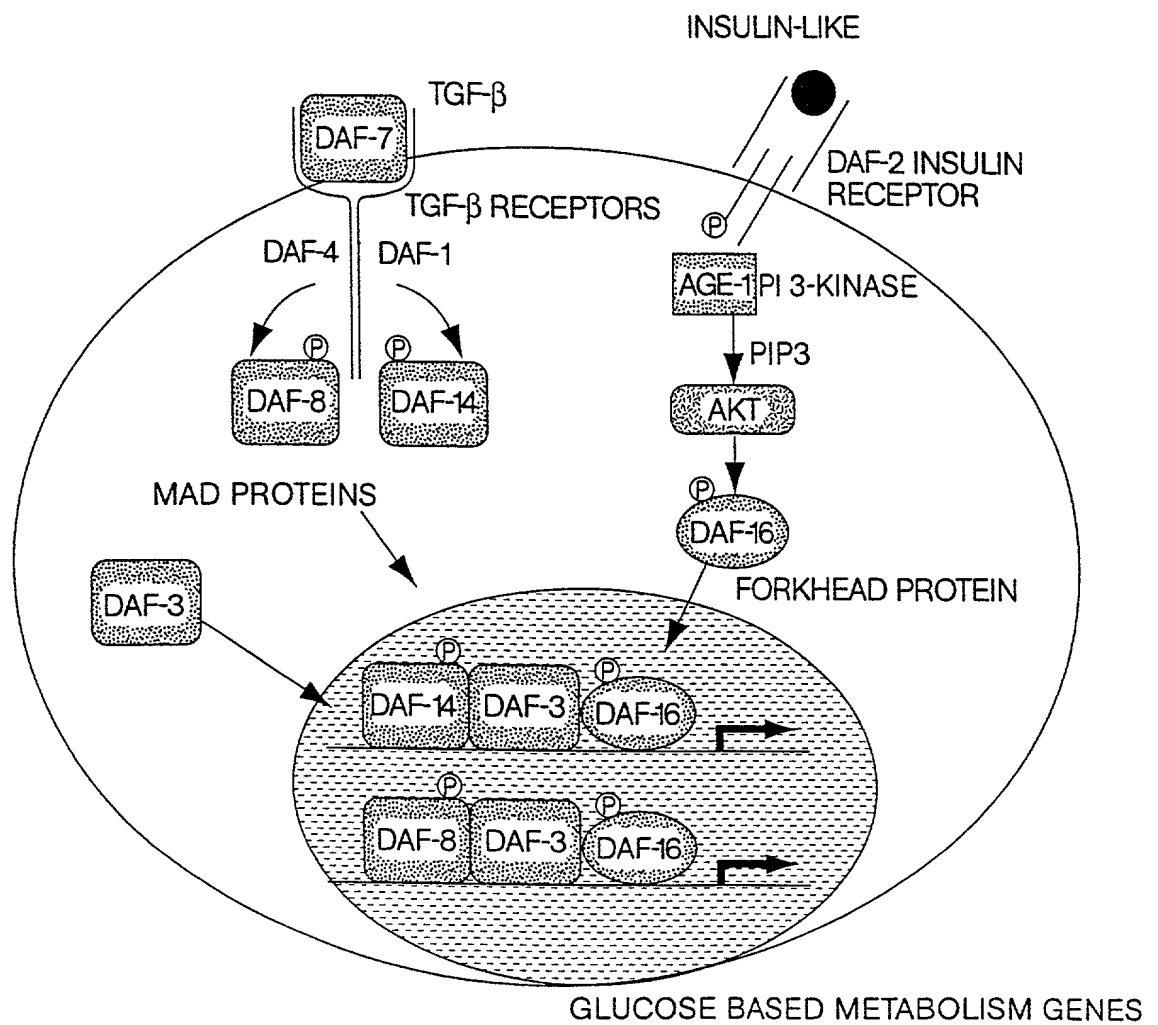
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 2821 aatacagcag ttcggagtat tgatccttcg tttatgaata agtggattcg gaaacaatgc  
 2881 ggaattgaag atgaaaagaa gaaaagcaaa aaggactcta cgaaaaatcc catcgaaaag  
 2941 aagattgata atactcaagc catgaagaaa tattttgaaa gtgtcgatcg attcctatac  
 3001 tcgtgtgttg gatattcagt tgccacgtac ataatgggaa tcaaggatcg tcacagtgat  
 3061 aatctgatgc tcaactgaaga tggaaaatat gtccacattg atttcgggtca cattttggga  
 3121 cacggaaaga ccaaacttgg gatccagcga gatcgtcaac cgtttattct aaccgaacac  
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 3241 ttcaaaacgt tatgcgtcga agcctacgaa gtaatgtgga ataatcgaga tttgttcggt  
 3301 tccttggttca ccttgatgct cggaatggag ttgcctgagc tgtcgacgaa agcggatttg  
 3361 gatcatttga agaaaaccct cttctgcaat ggagaaagca aagaagaagc gagaaagttt  
 3421 ttcgctggaa tctacgaaga agccttcaat ggatcatggt ctacccaaac gaattggctc  
 3481 ttccacgcag tcaaactacta ctga

Fig. 15 (sheet 2 of 2)

1 RKPWSSRSDC WTRTELRRIS QMHVNILHPQ LQTMVEQWQM RERPSLETEN GKGSLLLENE  
 61 GVADIITMCP FGEVISVVFP WFLANVRTSL EIKLSDFKHQ LFELIAPMKW GTYSVKPQDY  
 121 VFRQLNNFGE IEVIFNDDQP LSKLELHGTF PMLFLYQPDG INRDKELMSD ISHCLGYSLD  
 181 KLEESLDEEL RQFRASLWAR TKKTCLTRGL EGTSHYAFPE EQYLCVGESC PKDLESKVKA  
 241 AKLSYQMFWR KRKAEINGVC EKMMKIQIEF NPNETPKSL HTFLYEMRKL DVYDTDDPAD  
 301 EGWFLQLAGR TTFVTNPDVK LTSYDGVRSE LESYRCPGFV VRRQSLVLKD YCRPKPLYEP  
 361 HYVRAHERKL ALDVLSVSD STPKQSKNSD MVMTDFRPTA SLKQVSLWDL DANLMIRPVN  
 421 ISGFDFPADV DMYVRIEFSV YVGTLTASK STTKVNAQFA KWNKEMYTFD LYMKDMPPSA  
 481 VLSIRVLYGK VKLKSEEFV GWVNMSLTDW RDELROGQFL FHLWAPEPTA NRSRIGENGA  
 541 RIGTNAAVTI EISSYGGVR MPSQGQYTYL VKHRSTWTET LNIMGDDYES CIRDPGYKKL  
 601 QMLVKKHESG IVLEEDEQRH VMMWRRYIQ QEPDLLIVLS ELAFVWTDRE NSELYVMLE  
 661 KWKPPSVAAA LTLLGKRCTD RVIRKFAVEK LNEQLSPVTF HLFILPLIQA LKYEPRQSE  
 721 VGMMLLTRAL CDYRIGHRLF WLLRAEIAL RDCDLKSEY RRISLMEAY LRGNEEHIKI  
 781 ITRQVDMVDE LTRISTLVKG MPKDVATMKL RDELRSISHK MENMDSPLDP VYKLGEMIID  
 841 KAIVLGSAKR PLMLHWKNKN PKSDLHLPC AMIFKNGDDL RQDMLVLQVL EVMDNIWKAA  
 901 NIDCCLNPYA VLPMGEMIGI IEVVPNCKTI FEIQVGTGFM NTAVRSIDPS FMNKWIRKQC  
 961 GIEDEKKKSK KDSTKNPIEK KIDNTQAMKK YFESVDRFLY SCVGYSVATY IMGIDRHS  
 1021 NLMLTEDGKY VHIDFGHILG HGKTKLGIQR DRQPFILTEH FMTVIRSGKS VDGNSHELQK  
 1081 FKTLCEAYE VMWNNRDLFV SLFTLMLGME LPELSTKADL DHLKKTLCN GESKEEARKE  
 1141 FAGIYEEAFN GSWSTKTNL FHAVKHY

Fig. 16

**CONVERGENT TGF- $\beta$  AND INSULIN SIGNALING  
ACTIVATE GLUCOSE-BASED METABOLISM GENES**



**Fig. 17**

IN PHEROMONE, NO TGF $\beta$  OR INSULIN-LIKE SIGNALS  
CAUSES REPRESSION OF ANABOLIC GENES

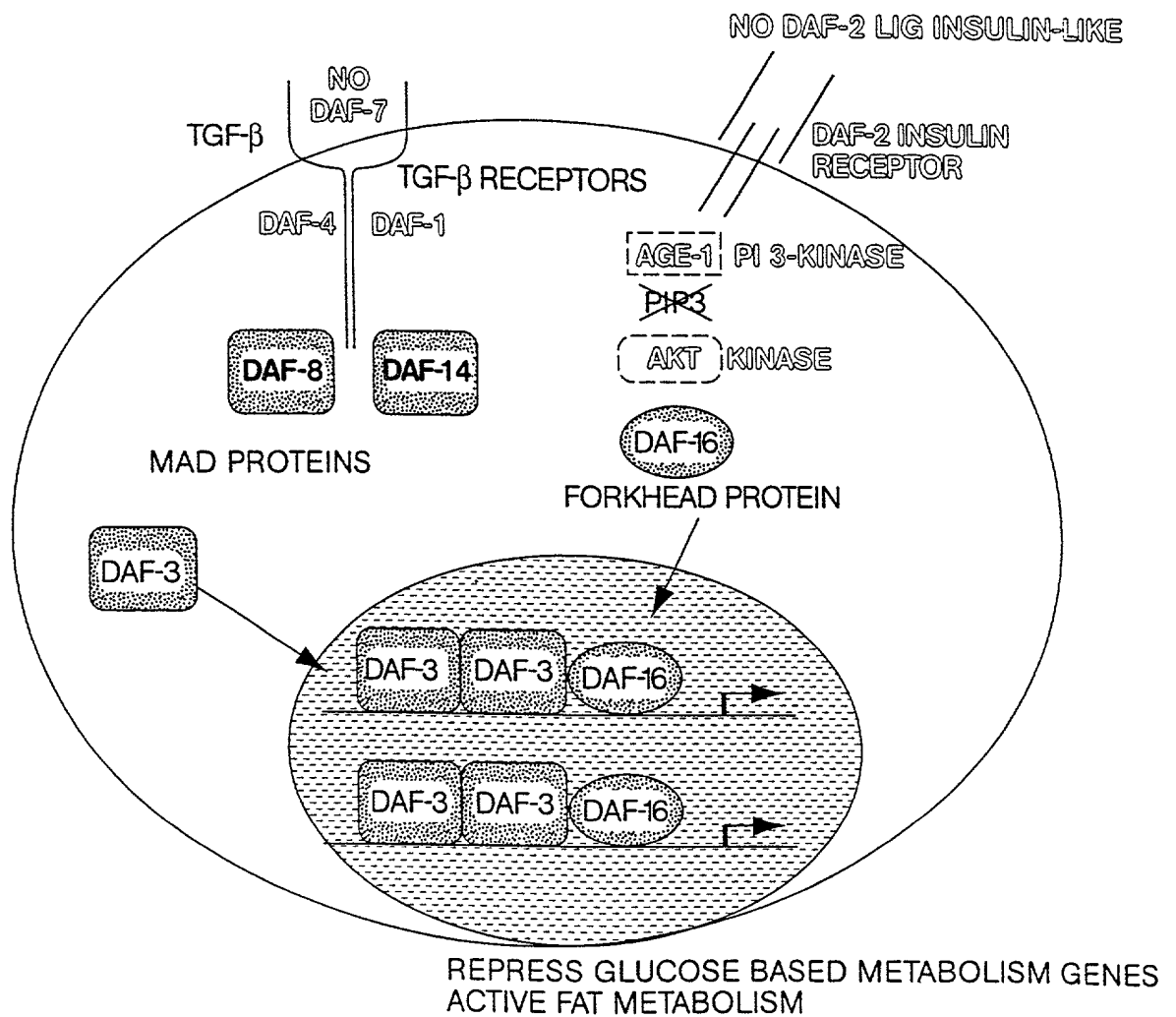
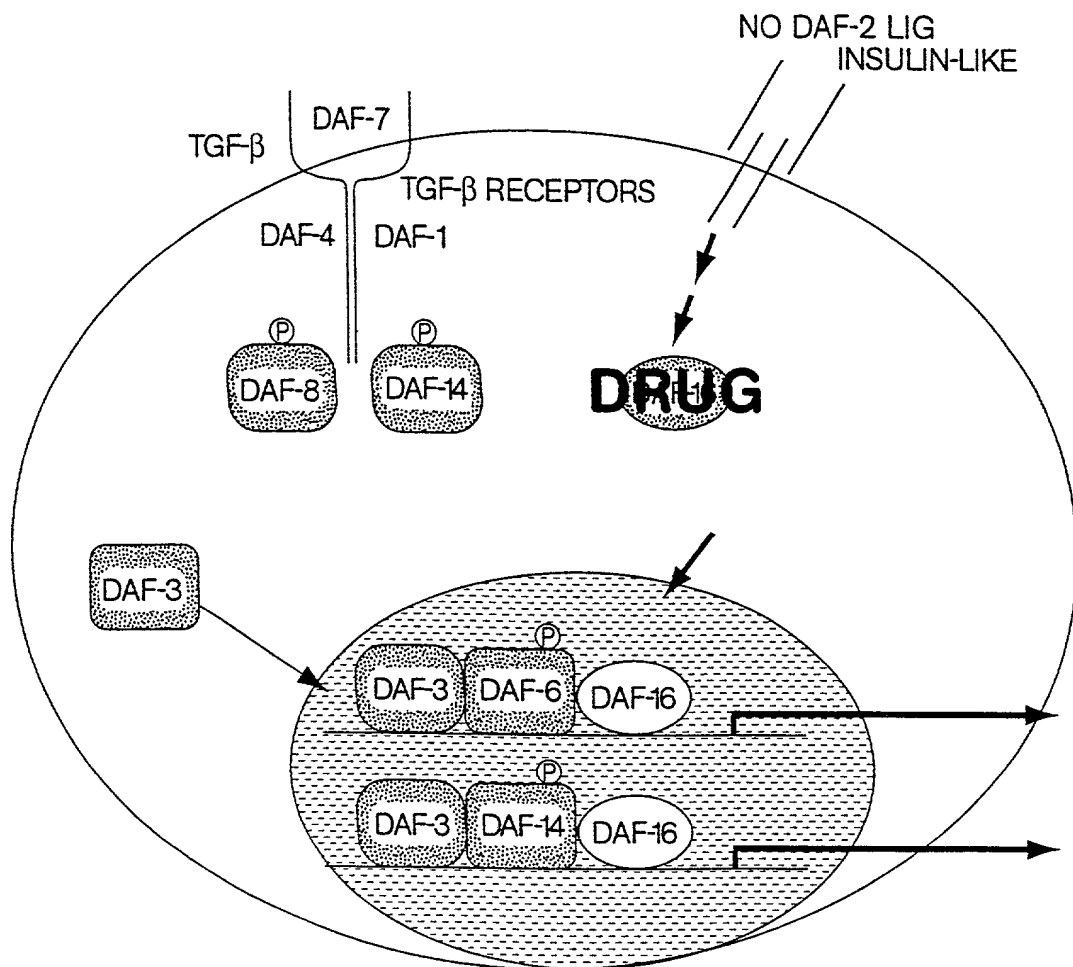


Fig. 18

DRUGS THAT INHIBIT DAF-16 OR DAF-3  
(OR PROTEINS IN THE PATHWAY)  
CAN BE DISCOVERED USING REPORTER GENES  
BEARING THEIR COGNATE BINDING SITES



DRUG CAUSES A DECREASE IN DAF-16 ACTIVITY, ACTIVATING  
THE REPORTER GENE LIKE A DAF-16 MUTANT.  
THIS BYPASSES THE NEED FOR INSULIN

Fig. 19

**DRUGS THAT INHIBIT DAF-3 WILL CURE  
THE DIABETES CAUSED BY A LACK OF DAF-7**

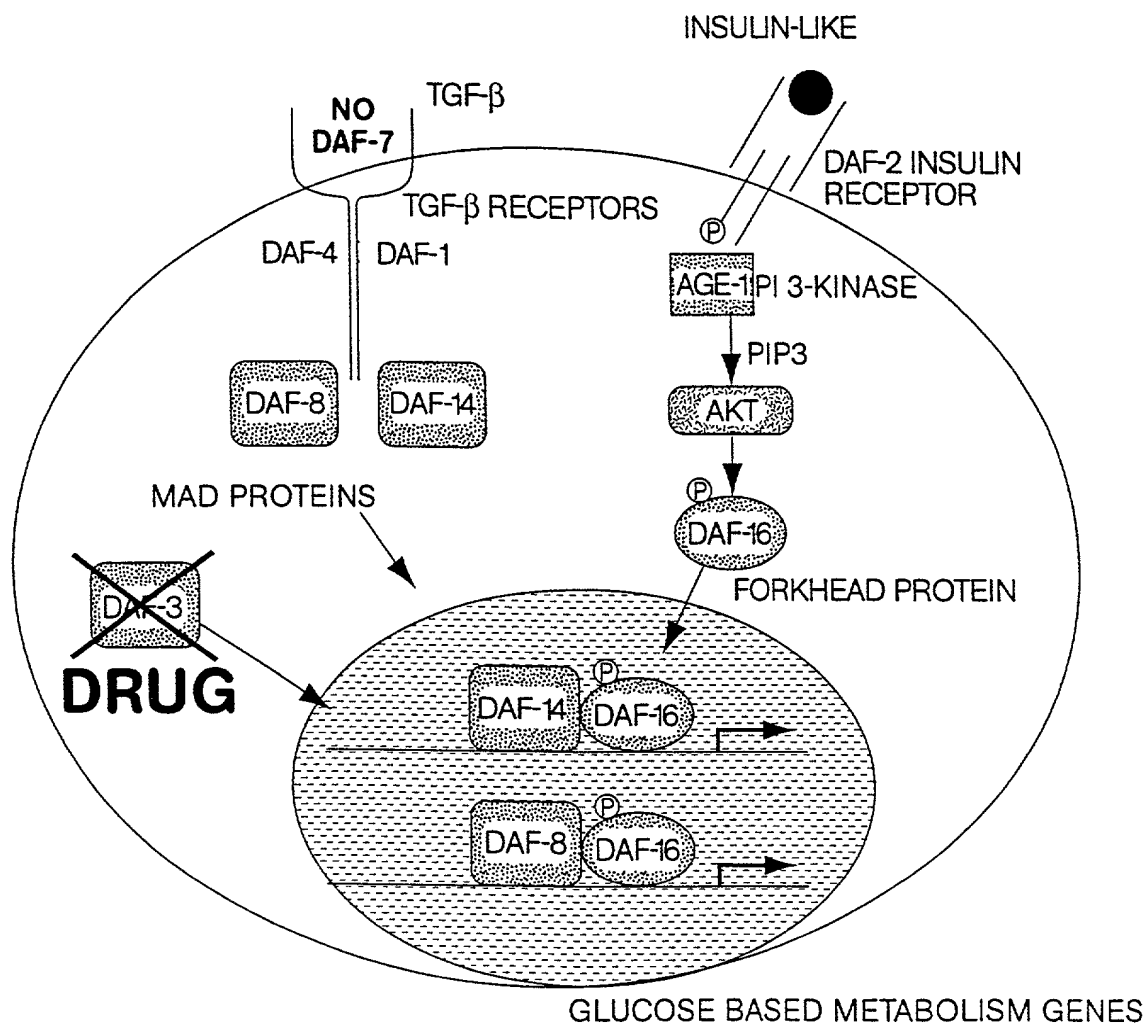


Fig. 20

DAF-16a1	1	MMEMLVDDQQGMDASSASTSTTSVSRFGADTFMNTD	DDVMMNDDMEFIPDRDR
DAF-16b	1	~MNDSDIDDDFPPEPRGRCTWPMQOYTYCESATTFPHHLNOHNNEYPHMHQLEPHMQQLPEOPLLN	~PDAAGLEPSASA
FKHR	1	~MAEAPQVVEIDPDEFLEPRRSCTWPLRPRPESQNSATSSPAPSGSAAAN	~EEDDEDDGGG
FKHRL1	1	MAEAPASPAPLSELEVELDPEFEFQSRPRSCWTPLQRP	ELQASPAKPSGETAADSMIFE
AFX	1	~	~MRIQEPQKAA
DAF-16a1	52	CN..TWPMRRPQLEPPLNSSPIIHEQIPEEDADLYGSNEQ..	CGQLGCASSNGSTAMLHTPDGSMNSHQTSFSPSDFRMSE
DAF-16b	68	LMNTLTSSGSSVASSIGGCAQCSEPCASGSSSTAATNSSQQOQOQTVGQMLAASVPCSSSGSTLGMNLNLSQGGGPM	PAPAKKKR
FKHR	64	AAVSADFMNSLSTLEESDFFQAPGSAVAAVAAAATAATGGLCGDFQCEACGL	LEHAPFPQPPFPGLSQHPPEVPPAA
FKHRL1	72	RAGSAMAIAGGGGSGTGLSGCLLEDS..ARVLAFCGQDPGSCPATAGGLSGGT	QALLQPOQPLP...
AFX	10	AIIDLDEDFEQSRPRSCWTPLRPRELEIANQPSPEPEVEPDLCEKVVHTEGRSEPI	LLPSRLDSEFAGCP...QPGILGAVT
DAF-16a1	127	SFDDTVSGCKTTTERRNAGNMSYAEELITTAIMASPEKRLTLAQVYEWMVQNVYFRRDKGDSNSSAGWKN	SIRHNLSLHSR
DAF-16b	148	CRKKP.TDQLAQKKNPWGEEYSYDITIAKALESAPDGRLLKLNELIYQMFSDN	NPYFGRSSPEEAAGWKN
FKHR	143	GPLAQPRKSSSRRNAGNLSYADLITKALESAPAEKRLTLSQIYEWNVKSVYFGRDKGDSNSSAGWKN	SIRHNLSLHSK
FKHRL1	143	C..SQPRK.CSSRRNAGNLSYADLITRAIESSPDKRLTLSQIYEWNVRCVPYFKDKGDSNSSAGWKN	SIRHNLSLHSR
AFX	86	GPRKG...GSSRRNAGNQSYAEFISQALESAPAEKRLTLAQIYEWNVRTVPYFKDKGDSNSSAGWKN	SIRHNLSLHSK
DAF-16a1	207	FMRIQNEGAGKSSWWVINPDAKPCGRNPRRTRRSNTIETTTKAQLEKSRRCAGKRRIKERALMGSLHSTL	NCNSIAGSIQT
DAF-16b	227	FMRIQNEGAGKSSWWVINPDAKPCGRNPRRTRRSNTIETTTKAQLEKSRRCAGKRRIKERALMGSLHSTL	NCNSIAGSIQT
FKHR	223	FVRVQNEGTGKSSWWMINPEG..GKSGKSPRRRAASMDNNSKFAKRSRAAKKK	.....AS.LQSGQEGA.GDSPGSGQ
FKHRL1	220	FVRVQNEGTGKSSWWMINPEG..GKSGKAPRRRAVSMVNSNKYTKSGRRAAKKK	.....AA.LQTAPESA.DDSP.SQ
AFX	160	FIRVHNEATGKSSWWMINPEG..GKSGKAPRRRAASMDSSSKLLRGRSKAPKKK	.....PSVLPAPEGATPTSPVGVH
DAF-16a1	287	ISHDLYDDDSMQGAFDNVPSFRPRRTQSNLSIPGSSSRVSPAIGSDIYDDL	EFPSWVGESVPAIPSDIVDRTDQMRIDA
DAF-16b	307	ISHDLYDDDSMQGAFDNVPSFRPRRTQSNLSIPGSSSRVSPAIGSDIYDDL	EFPSWVGESVPAIPSDIVDRTDQMRIDA
FKHR	292	FSKWPAPEGSHSNDFFDNWSTFRPTSSNAS..TISGRLSPIM..TEQDDDLGEGD	.VHSMVYPPSAAKMAST.....
FKHRL1	288	LKWPGPSPTSRSDELDAWTFRSRTSNAS..TVSGRLSPIMASTELDEVQDDDDAPLSPMLYSSSASLSPSVSKPCTVE	
AFX	231	FAKWSGSPCSRNRREADMWTTFRPRSSNAS..SVSTRLSPRPESEV.LAEIIPASVSSYAGGVPTLNEGLELLDGLN	
DAF-16a1	366	THHIGGVQIKQESKPIKTEPIAPPSPSYHELNSVRGSCAQNPLLRNPIVPSTNEFKPMPLPGAY	CNYQNGGGITPINWLSTSN
DAF-16b	386	THHIGGVQIKQESKPIKTEPIAPPSPSYHELNSVRGSCAQNPLLRNPIVPSTNEFKPMPLPGAY	CNYQNGGGITPINWLSTSN
FKHR	359	LPSSLSEISNPENM.ENLLDNL.NLLSSPTSLTVSTQSSPGTMMQQTCCYSEAPP	NTSLNSPSPNYQKTYGQSSMSPLP
FKHRL1	366	LPRLTDMAGTMNLDGLTENLMDDDLNDNITLPPSQPSPTGGLMQRSSSEFPYTTK	GSGLGSPSTSFNSTVFGPSSLSLR
AFX	308	LTSSHSLLSRSGLSGFSLQHPGVGTGPLHTYSSLSLSPAEGLPSAGEGCESSSQALEALTSDT	TPPPPADVLMTQVDPILS
DAF-16a1	446	SSPLPGIQS..CGIIVAAQHTVASSSALPIDLENLTLDPQPLMDTMDVDALIRHELSSQAGQHIFHFDL	~
DAF-16b	466	SSPLPGIQS..CGIIVAAQHTVASSSALPIDLENLTLDPQPLMDTMDVDALIRHELSSQAGQHIFHFDL	~
FKHR	436	QMPITQLQDNK..SSYGGMSQYNCAPELKELELTSDEPHNDI	MTVPDEGVAQPNRSVLQNV...MMGPN
FKHRL1	445	QSPMQTIOENKPAFTSSMSHY..GNQTLQDLTLTSDLSLSDVMMTQSDPTMSQASTAVSAQNSRRNVML	LRNDPMMSFAAQ
AFX	388	QAPTLLLLLGLPSSJ...SKLATGVGLCPKPLEARGEFS	SSLVPTLSMIAPPVPMASAPIPKALCTPVLTTPTEAASQDRMP
DAF-16a1	511	~	~
DAF-16b	531	~	~
FKHR	511	ASHKNMNPSSH.THPGHAQOTSAVNGRPIPHPTVSTMPTHTSGMNRRLTQVKT	PVQVPLPHPMQMSALGYSVSSCNGYGR
FKHRL1	523	PNQGSLVN.QNL.LHHQHQTQGALGSRALNSVSNM.GLSESSLSGSAHQOQ	QSEVVSQSMQ.TLSDLSGSSLYSTSAN
AFX	464	QDLDDLMYMENTLECDMDNIIISDLMDEGEGLDNFEPDE	~

**FIG. 21A-1**



# Fork head Domain Alignment (*C. elegans*, human, others)

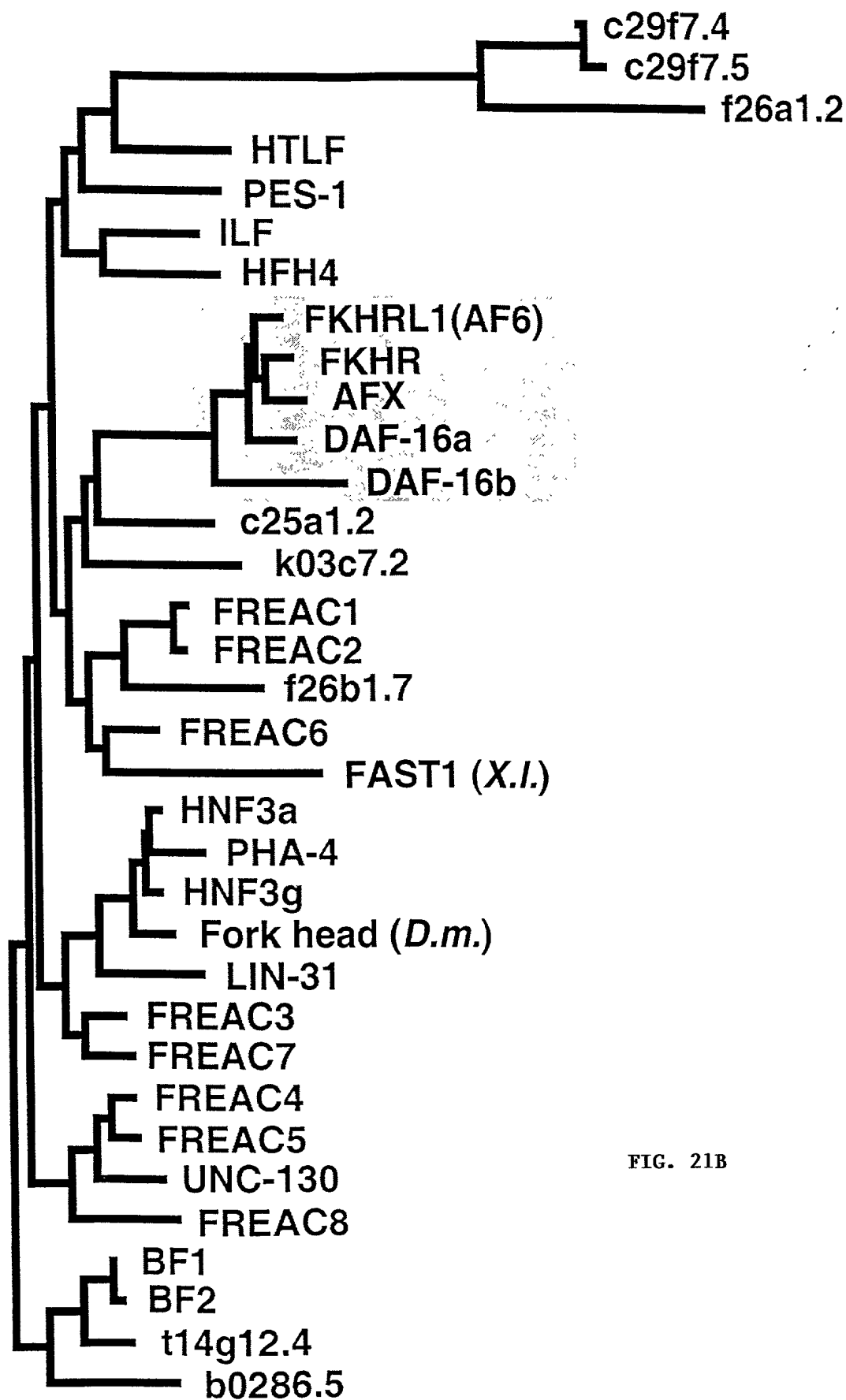


FIG. 21B

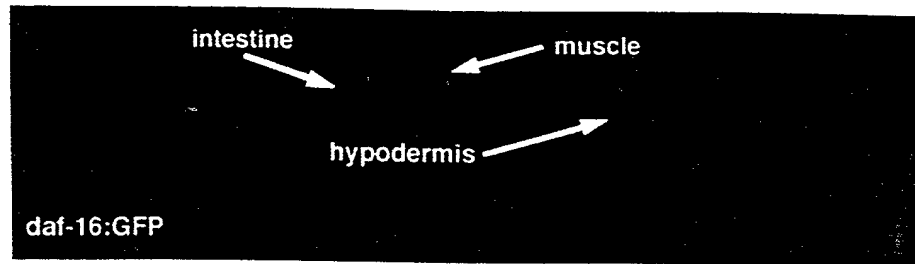


Fig. 22

# INJECTION OF OF DAF-7 BYPASSES OBESITY-INDUCED DEFECTS IN INSULIN-REGULATION OF METABOLISM

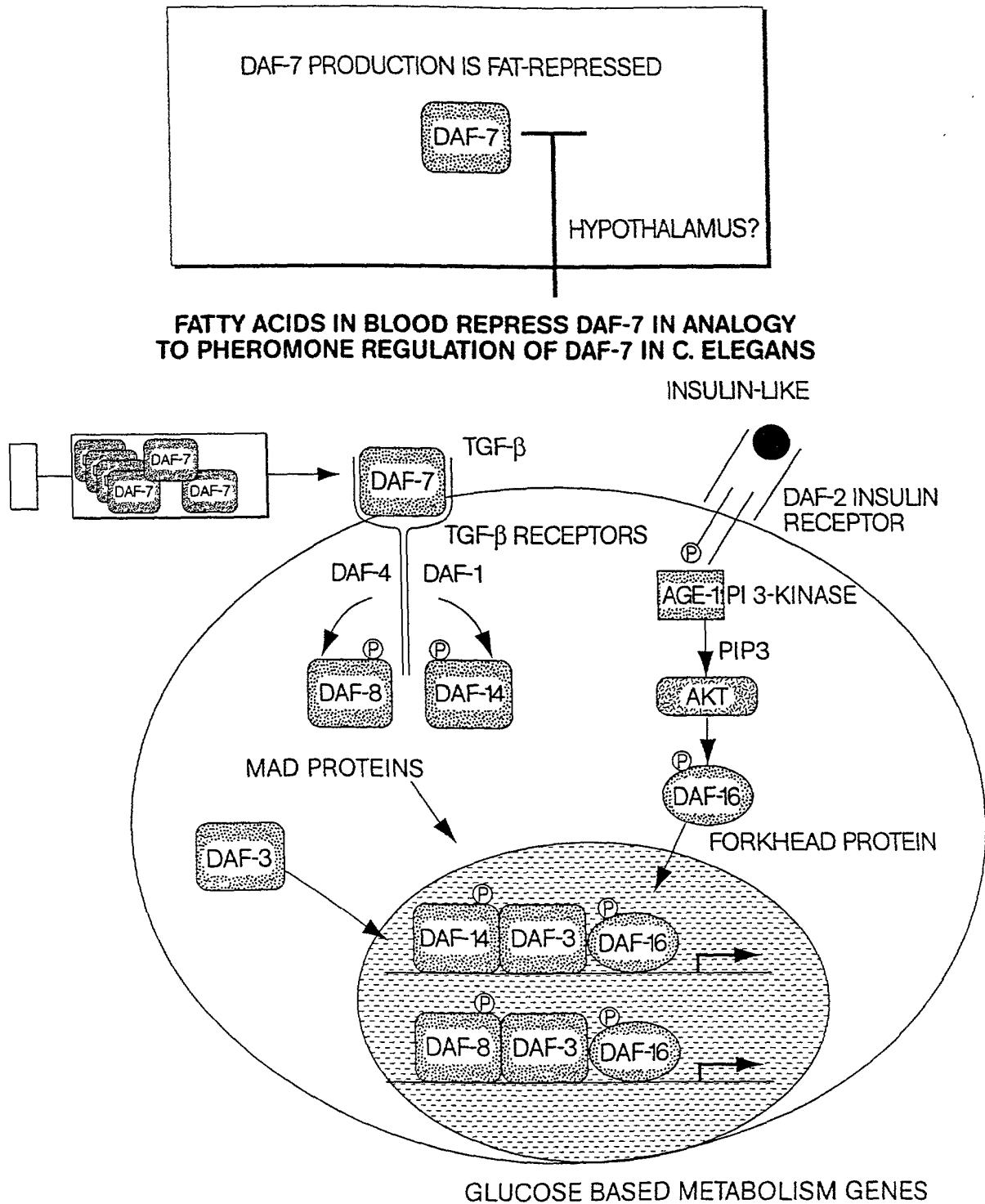


Fig. 23

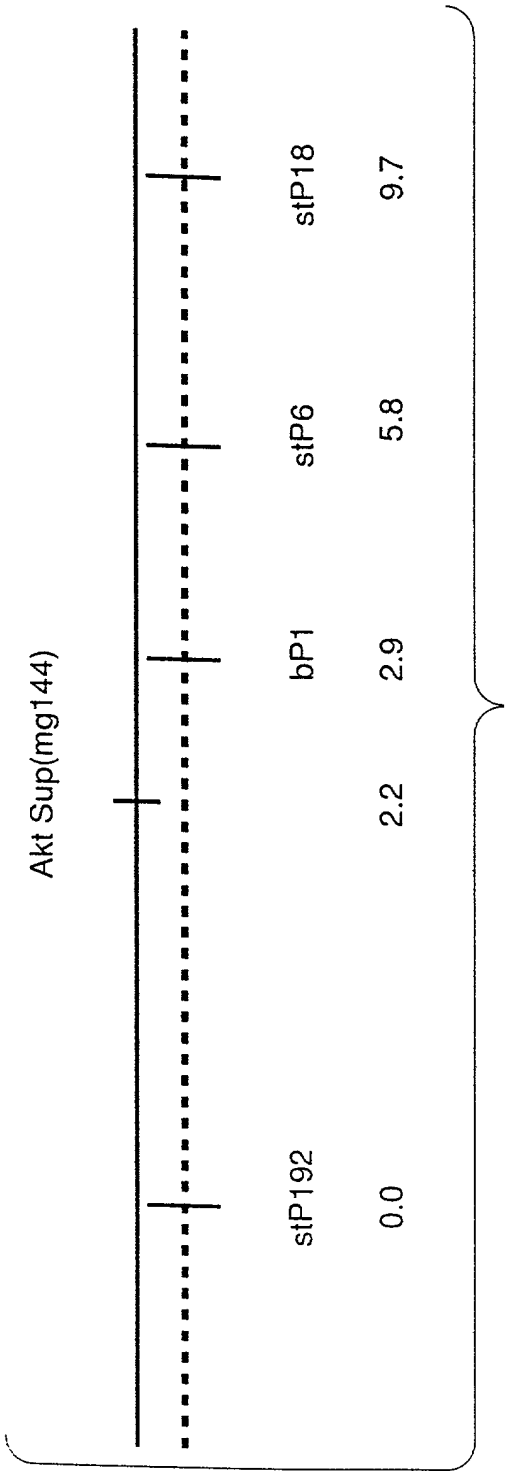


Fig. 24

Comparison of the human AKT protein sequence to the cosmid sequence C12D8, located in the genetic interval where sup(mg144) maps. Numbering in the AKT protein sequence by amino acid residues, and in the cosmid sequence by nucleotide position.

Score = 450 (207.4 bits), Expect = 5.2e-165, Sum P(7) = 5.2e-165  
Identities = 79/121 (65%), Positives = 97/121 (80%), Frame = +1

Query: 319 EVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFELILMEEIRFPRTLGPPEAKS 378  
+VL+D+DYGR VDWNG+GVVMYEMMCGRLPFY++DH KLFELI+ ++RFP L EA++  
Sbjct: 33685 QVLDDHDYGRCDWNGVGVVMYEMMCGRLPFYSKDHNLKLFELIMAGDLRFP SKLSQEART 33864

Query: 379 LLSGLLKKDPTQRLGGGSEDAKEIMQHRFFANIVWQDVYEKKLSPPFKPQVTSETDTRYFD 439  
LL+GLL KDPTQRLGGG EDA EI + FF + W+ Y K++ PP+KP V SETDT YFD  
Sbjct: 33865 LLTGLLVKDPTQRLGGGPEDALEICRADFFRTVDWEATYRKEIEPPYKPNVQSETDTSYFD 34047

Score = 256 (118.0 bits), Expect = 5.2e-165, Sum P(7) = 5.2e-165  
Identities = 48/66 (72%), Positives = 59/66 (89%), Frame = +1

Query: 146 TMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVAHTLTENRVLQNS 205  
TM +F++LK+LGKGTFGKVIL KEK T + YA+KILKK+VI+A++EVAHTLTENRVLQ  
Sbjct: 32314 TMEDFDLKVLGKGTFGKVILCKEKRTQKLYAIKILKDVIIAREEVAHTLTENRVLQRC 32493

Query: 206 RHPFLT 211  
+HPFLT  
Sbjct: 32494 KHPFLT 32511

Score = 190 (87.6 bits), Expect = 5.2e-165, Sum P(7) = 5.2e-165  
Identities = 36/45 (80%), Positives = 37/45 (82%), Frame = +2

Query: 276 KLENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEV 320  
KLENL+LDKDGHIKI DFGLCKE I G TFCGTPEYLAPEV  
Sbjct: 33509 KLENLLLDKDGHIKIADFGLCKEEISFGDKTSTFCGTPEYLAPEV 33643

Score = 188 (86.7 bits), Expect = 5.2e-165, Sum P(7) = 5.2e-165  
Identities = 37/57 (64%), Positives = 42/57 (73%), Frame = +3

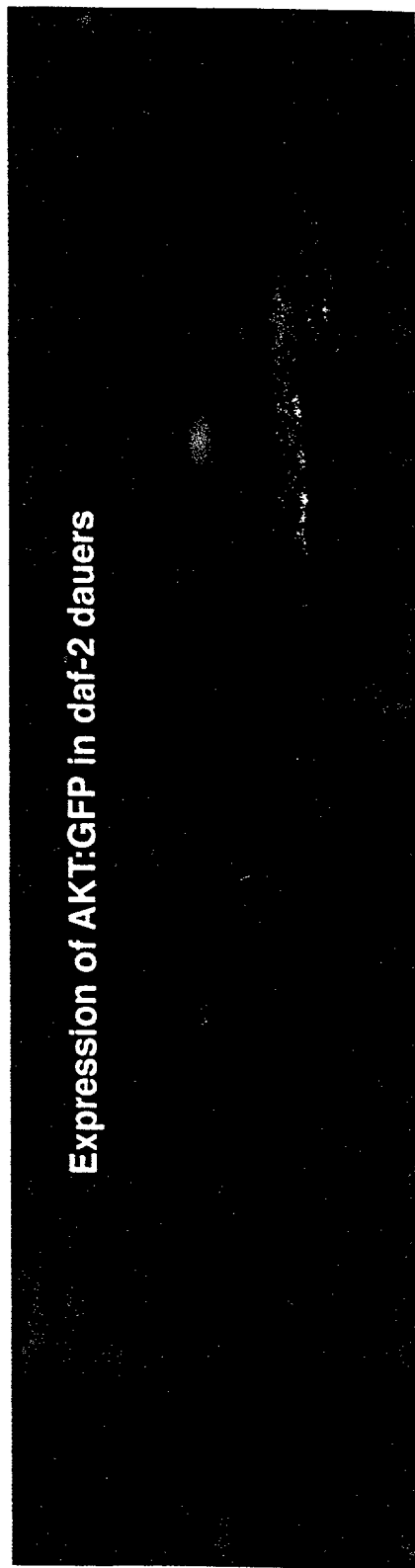
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+ LKYSFQ LCFVM++ANGGELF H+ + FSE RARFYGAIEIV AL YLH  
Sbjct: 32667 YFQELKYSFQEQHYLCFVMQFANGGELFTHVRKCGTFSEPRARFYGAIEIVLALGY LH 32837

Score = 166 (76.5 bits), Expect = 5.2e-165, Sum P(7) = 5.2e-165  
Identities = 29/59 (49%), Positives = 42/59 (71%), Frame = +1

Query: 53 NNFSVAQCQLMKTERPRNTFFIIRCLQWTTVIERTFHVETPEEREWATAIQTVDGLK 111  
+ F++ Q M E+PRPN F++RCLQWTTVIERTF+ E+ E R+ W AI++++ K  
Sbjct: 31846 STFAIFYFQTMLFEKPRPNMFMVRCLQWTTVIERTFYAESAEVRQRWIHAIESISKKYK 32022

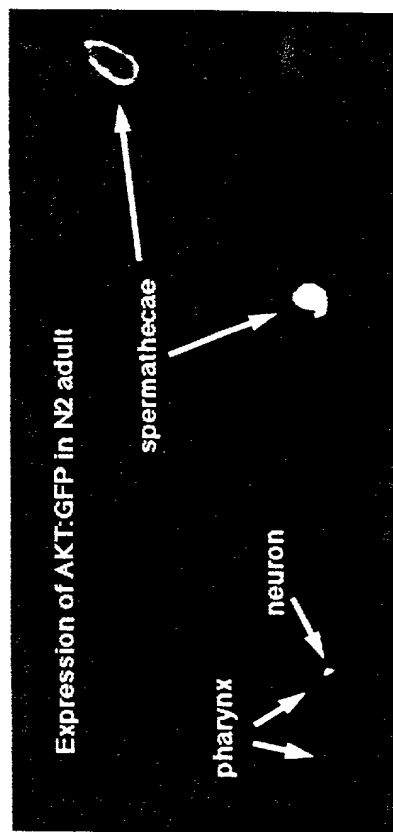
Score = 134 (61.8 bits), Expect = 5.2e-167, Sum P(8) = 5.2e-167  
Identities = 24/33 (72%), Positives = 30/33 (90%), Frame = +3

Query: 210 LTALKYSFQTHDRLCFVMEYANGGELFFHLSRE 242  
L LKYSFQT+DRLCFVME+A GG+L++HL+RE  
Sbjct: 33156 LQELKYSFQTNDRLCFVMEFAIGDLYYHLNRE 33254



Expression of AKT:GFP in daf-2 dauers

Fig. 26A



Expression of AKT:GFP in N2 adult

spermathecae

pharynx

neuron

Fig. 26B

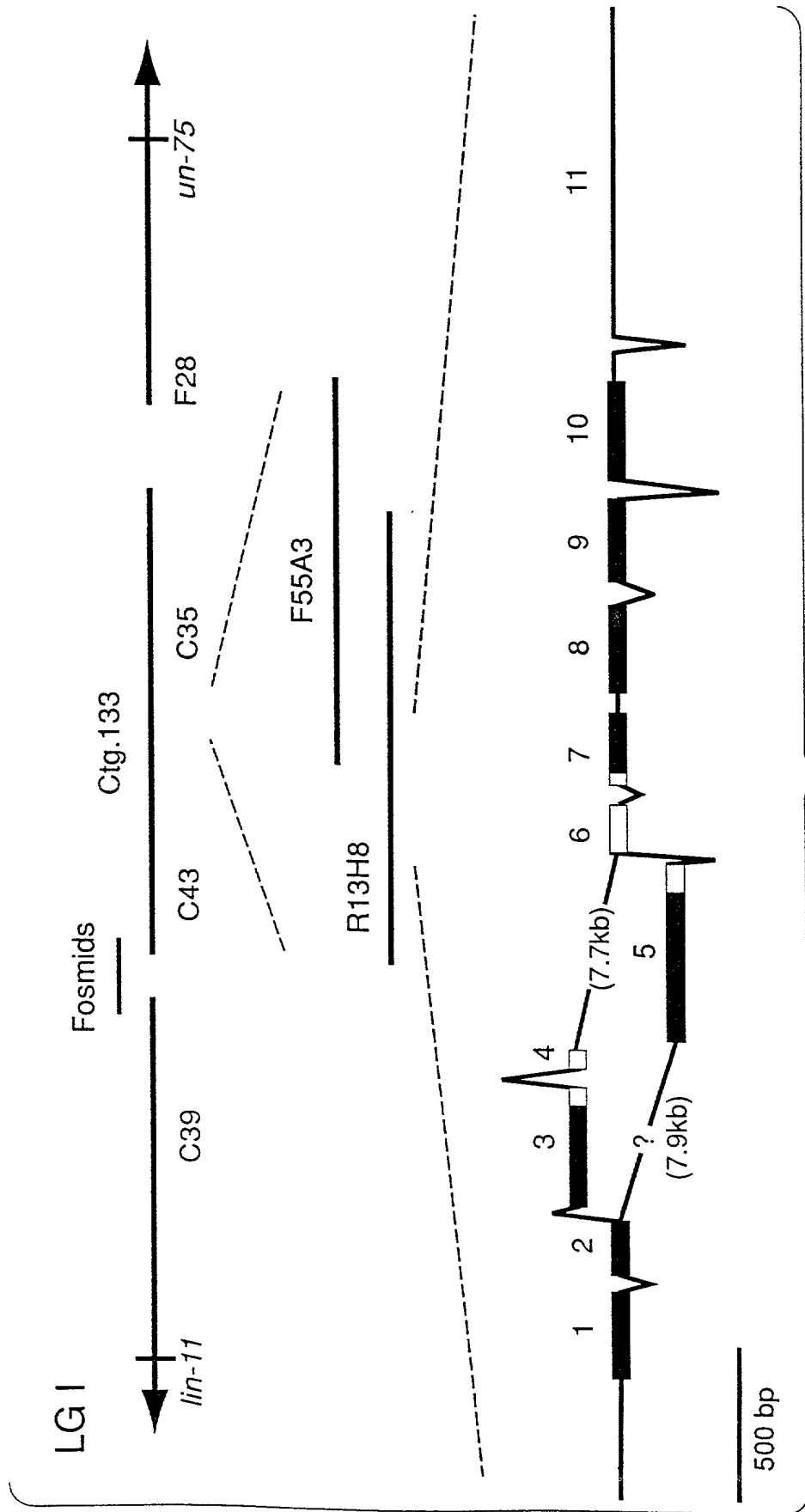


Fig. 27

	1	15 16	30 31	45 46	60	
1 ZK84.6	-MNSVFTIIFVLCAL	QVAASFRQSG	---P	SMSEESASMQLLREL	QH--NMESAHRPMP	54
2 ZK75.1	-MFSFFT-YFLLSAL	LLSASCRC	-----P	SMDT-SKADRILREI	E---METELENQLS	47
3 ZK1251.2	----MPPIILVFFLV	LIPASQQY	-----P	FSLE-SLNDQIINEE	VI--EYMLENSIRSS	47
4 C06E2	--MIVTLIVFLVIGL	QMAHLSQVSGNNENG		FLNP-FDLSQWSEEI	LHRQYHHHHHHHHGN	57
5 ZK75.2	----MNAIIFCLLET	TVTATYEVF	-----G	KGIEHRNEHLIINQL	D---IIPVESTPTPN	48
6 ZK75.3	MKLSVVLALFIIFQL	GAASLMRN	-----W	MFDFEKELEHDYDDS	E---IGFHNHSLMA	51
7 C17C3	-----	-----		-----MKLLHI	F---IIFLLFQSCSN	18
8 F13B12	-----	-----		-----MYWFRQVYRPS	FF--FGFLAILLLSS	50
9 INSULIN	-----	-----		-----MA	LWMRLPLALLALW	17
CONSENSUS	-----	-----		-----	-----	

	61	75 76	90 91	105 106	120	
1 ZK84.6	RARRVPAPGETRACG	RKLISLVMVAVCGD-L	CN-----	-----	-----	85
2 ZK75.1	RARRVPA-GEVRACG	RRLLLFVWSTCGE-P	CT-----	-----	-----	77
3 ZK1251.2	RTRRVPDEKKIYRCG	RRIHSYVFAVCGK-A	CE-----	-----	-----	78
4 C06E2	RARRTLETEKIYRCG	RKLYTDVLSACNG-P	CE-----	-----	-----	88
5 ZK75.2	RASRVQK---RLCG	RRLILFMLATCG--E	CD-----	-----	-----	74
6 ZK75.3	RSRRGDK---VKICG	TKVLKMVMVMCGG-E	CS-----	-----	-----	79
7 C17C3	KMCQYSK-KKYKICG	VRALKHMKVYCTR-G	MT-----	-----	-----	48
8 F13B12	PTPSDAS---IRLCG	SRLTTTLAVCRNQL	CTGLTAFKRSADQSY	APTTRDLFHIHHQQ-		80
9 INSULIN	GPDPAAAFVNQHLCG	SHLVEALYLVCGERG	FFYTPKTRREAEDLQ	VGQVELGGGPGAGSL		77
CONSENSUS	-----CG	-----C	-----	-----	-----	

B CHAIN

C PEPTIDE

	121	135 136	150 151	165 166	180
1 ZK84.6	-----PQEGKDIA	TECCGNQCSDDYIRS	ACCP-----	112	
2 ZK75.1	-----PQEDMDIA	TVCCTTQCTPSYIKQ	ACCPEK---	106	
3 ZK1251.2	-----SNTEVNIA	SKCCREECTDDFIRK	QCCP-----	105	
4 C06E2	-----PGTEQDLS	KLCCGNQCTFVEIRK	ACCADKL--	118	
5 ZK75.2	-----TDSSDLS	HICCIKQCDVQDIIR	VCCPNSFRK	106	
6 ZK75.3	-----S-TNENIA	TECCEKMCTMEDITT	KCCPSR---	107	
7 C17C3	-----R-DYGKLL	VTCCSKGCNAIDIQR	ICL-----	73	
8 F13B12	-----KRGGIA	TECCEKRCSFAYLKT	FCCNQDDN-	109	
9 INSULIN	QPLALEGSLQKRGIV	EQCCTSICSLYQLEN	YCN-----	110	
CONSENSUS	-----	-----CC---C	-----C	-----	

A CHAIN

Fig. 28

Zk75-1	ACGRRRL	WST	xxQE	DIAT	VCC	CTPS	YTKQA	46
Zk84-6	Acgrk	ma	vcgdl	cnx	ecgnq	csdd	Yrsac	46
Zk1251-2	RCGRR	FAV	CGKAC	EX	KCCRE	CTDD	YIRKQ	46
C06e2	RCGRK	LSA	CNGP	CEx	LCCGN	CTFV	YIRKAC	46
Zk75-3	ICGTR	MVM	CGEC	SX	ECCEK	CTME	YITTK	46
Zk75-2	lgrri	lat	cgcd	tx	ICCIK	cdvq	Yirvc	46
Ins-Human	LCGSH	YLV	CGER	GFX	CCTSI	CSLY	YENY	46
Ins-Rabbit	lgrsh	Ylv	cggrg	fx	ccctsi	csly	Yleny	46
Ins1-Xenopus	lgrsh	Ylv	cgdr	fx	ccchst	csly	Ylesy	46
Ins2-Xenopus	lgrsh	Ylv	cgdr	fx	ccchst	csly	Yleny	46
Ins-Alligator	lgrsh	Ylv	cgdr	fx	ccchnt	csly	Yleny	46
Ins-Elephantfish	lgrsh	Ylv	cgdr	fx	ccchnt	csly	Yleny	46
Igf1-Bovine	LCGAE	QFV	CGDR	GFX	ECFFR	CDLR	YLEMY	46
Igf1-Dog	lgrae	qfv	cgdr	fx	ecffrs	cdlr	Ylemy	46
Igf2-Horse	lgrge	qfv	cgdr	fx	ecffrs	cdla	Ylety	46
Igf2-Human	LCGGE	QFV	CGDR	GFX	ECFFR	CDLA	YLETY	46
Ilp-Amphioxus	LCGST	SFV	CGNR	GYX	ECYNV	CDYS	YLESY	46
Lirp-Locust	YCGEK	KL	VCRGN	YNX	ECCKR	CSIS	ELQTY	46
Bxa4-Bommo	YCGRH	AD	CWEA	GVX	ECCLRP	CSVD	VLLSY	46
Bxb1-Bommo	YCGRH	AD	CFGE	KX	ECFRP	CTLD	VLLSY	46
Bxrpa-Hornworm	lgrh	ad	cpnv	eyx	ccvns	ctmd	Ylisy	46
Bxa1-Silkworm	Ycgr	lat	m	l	ecckp	cten	Ylisy	46
Bxa2-Silkworm	Ycgr	lat	m	l	ecckp	cten	Ylisy	46
Bax3-Silkworm	Ycgr	lat	m	l	ecckp	cten	Ylisy	46
F13b12	Ycgr	lat	m	l	ecckp	cten	Ylisy	46
Mpi3-Seasnail	LCGSR	QW	CS	TYTTX	ECCKR	CSFA	YLKT	46
Relaxin-Human	LCGST	QW	CS	TYTTX	ECCKR	CSFA	YLKT	46
Rlf-Human	LCGRE	IAI	CGM	STWX	ECCKR	CSFA	YLKT	46
	lgrh	vr	vcg	pprwx	ECCKR	CSFA	YLKT	46

Fig. 29

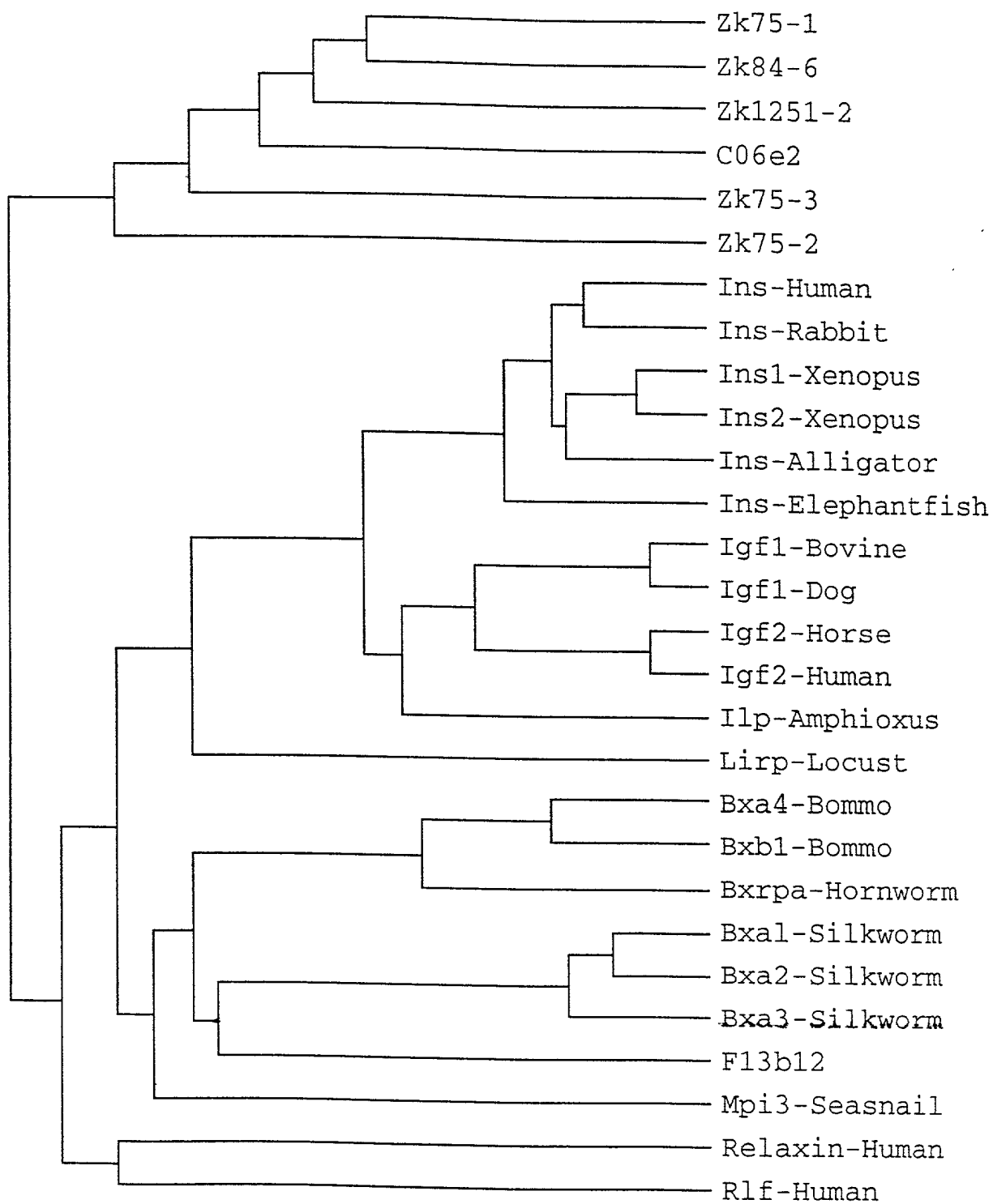


Fig. 30

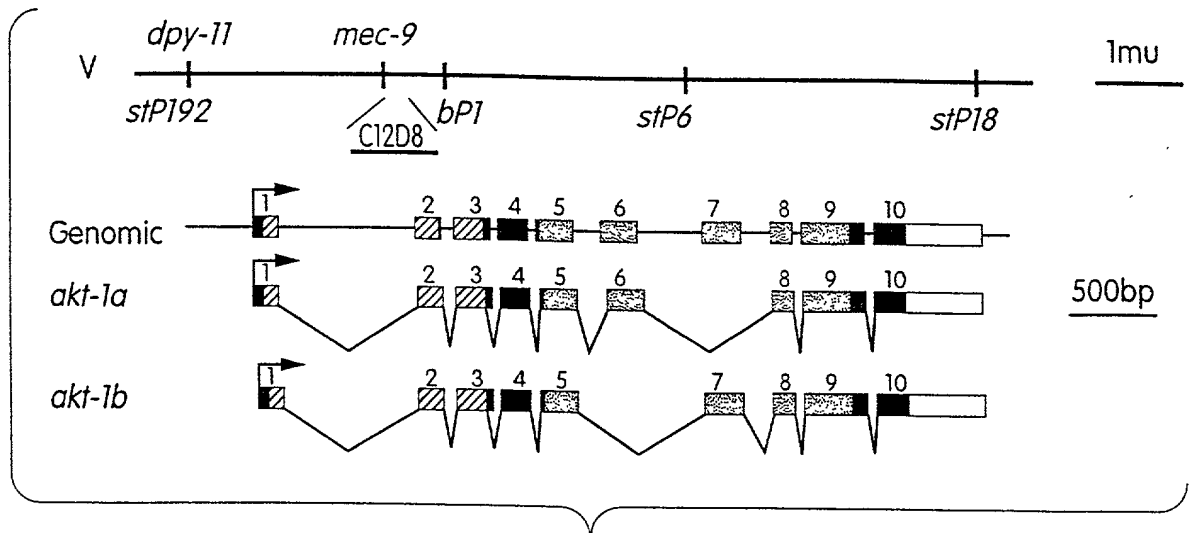


Fig. 31

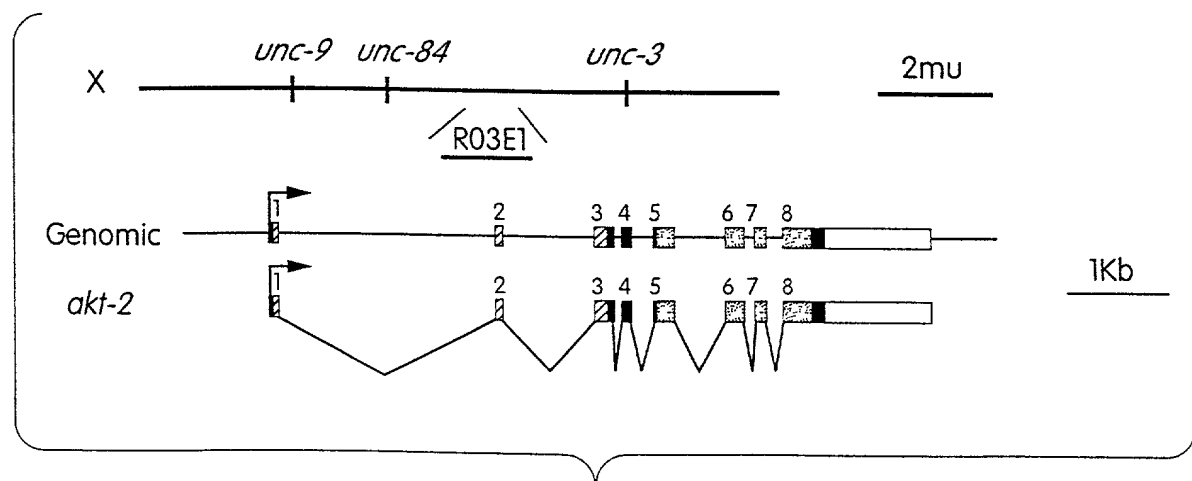


Fig. 32

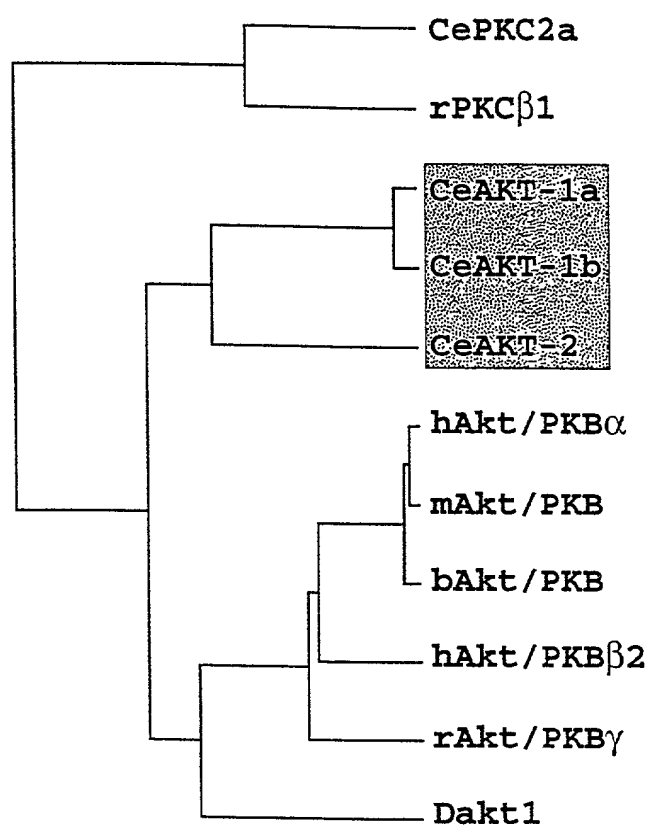


Fig. 33

AKT-1a MSMTSLSTKSRR--QEDVVI EGWLHKKGEHIRNWRPRYFMI FNDGALLGFRAKPKEGQPFPEPL  
 AKT-1b .....--  
 AKT-2 M..ENAHLOK..I...S.....IL.R..T.....S...D..L....  
 hAkt/PKBa MSDVAI K.....R..Y.KT.....LLK...TFI.YKER.QDVDQREA..

AKT-1a NDFMIKDAATMLEKPRPNMFMVRCLQWTTVIERTFYAESAEVRQRWIHAIESIS--KKYKGTN  
 AKT-1b .....  
 AKT-2 .N...R...VCLD.....I.....D..DF.....E...QAV.SHNRL..ENA  
 hAkt/PKBa .N.SVAQCQL.KT.R...T..II.....HV.TP.E.EE.TT...QTVADGL.KQE--  
 mg144 T

AKT-1a ANPQEELMETNQPKIDEDSEFAGAAHAIMGQPSSGHGDNCSIDFRASMISIADTSEAAKRDKI  
 AKT-1b .....  
 AKT-2 G.TSMQEED..GN.SGES.VNM-----DAT.TRS....ESTVMN.DEPE.VPRKNTV  
 hAkt/PKBa -----E.EMD.-----R.GSPS..SGAE-----EMEV.L.KPKHRV

AKT-1a TMEDFDLKVLEKGTGKVLCKEKRTOKLYATKLEKKDVTIAREEVAHTLETENRVLQCKHPF  
 AKT-1b .....  
 AKT-2 ..D.....Q.....R..SSD.....IR.EMVVD.S.....YA.V..  
 hAkt/PKBa ..NE.EY..L.....V...A.GRY..M.....E..V.KD.....NSR...

AKT-1a LTELKYSFQEQHYLCFVMQFANGGELFTHVRK-----CGTFSEPRAREFYGAELVLAEGYLH-RC  
 AKT-1b .....TNDR.....E..I..D.YY.LNREVQMKEG.....S.....-AN  
 AKT-2 ..L.....A.YHL.....E.....LQR-----K...A.T...S..I.....-HR  
 hAkt/PKBa ..A.....THDR.....EY.....F.LSRE-----RV..D.....S..D...SEK

AKT-1a DIVYRDMKLENLLDKDGHIKIADFGLCKEEISFGDKTSTFCGTPEYLAPVLDDHDYGRCDW  
 AKT-1b S.....L.....  
 AKT-2 N.....R.....T.....KY.....IE.I..D.S..  
 hAkt/PKBa NV....L.....M.....T.....G.KD.ATMK.....E.N...A...

AKT-1a WGVGVVMYEMMCGRLPFYSKDHNKL FELIMAGDLRFPSKLSQEARTLLETGLLVKDPTQRLGCGP  
 AKT-1b .....  
 AKT-2 .....SA.ENG.....TTC..K..NR..P..V...S...ERV.AK...A..  
 hAkt/PKBa ..L.....NQ...E.....LMEEI...RT.GP..KS...S...K...K.....S

AKT-1a EDALEICRADFERTVDWEATYRKEIEPPYKPNVQSETDTSYFDN-EFTSQPVQLTPPSRSGALA  
 AKT-1b .....  
 AKT-2 D..R.VS...E..KD.....L...V...F...M.....F..RVRYV.ILLKV-----E.I  
 hAkt/PKBa ...K...MOHR..AGIV.QHV.E.KLS..F..Q.T....R...E-...A.MITI...DQDDSM

AKT-1a TVDEQEEMQSNFTQFSFHNVMGSINRIHEASEDNEDYDMGZ  
 AKT-1b .....  
 AKT-2 .....  
 hAkt/PKBa C...--S.RRPH.P...YSASSTA

Fig. 34

cataaaatccagtaaattggtaaaattttcaatttcagatccatctcgatggaggatctcacaccaactaacacgtcgctcgacaccacaactac  
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Fig. 35A

at tt t t g g t a g g t t g a c a t g a a a c t t t a a a a c t g a a t a c g t a a t t t t c a a c t t a c a g g t g c g c g a c c c g a g t a c c c g t a t c a c c a g t c a a g a a c t  
t a t g g c t c a c a a g t t t t t t g a a a c g t t g a c t g g g t g a a c a t t g c a a a t a t c a a g c c a c c a g t c c t g c a c g c c t a c a t t c c a g c c a c a t t t g g c g  
a g c c g g a g t a c t a c t c t a a c a t t g g g c c t g t c g a g c c g g a c t t g a t g a t c G T G C C T T G T T C C G T T T G A T G A A T T T G G G A A A T G A T G C T A G C G C A  
T C A C A G C C A T C A A C G T G A G T T T G A A G C A T T T T T T C T T G C A T T A A A A G T T T T A C C T T G C A C T G A C C A A A T T T A T T G A A A C T A T T A A T T A T T T G A  
T T C T G A T T A A C A A T G A C C A A A A G A T T T G A A C T G A C A A A G T G C A A A T T T G C A C C G A C C A A A A A C A G T T T G C A C T G A C C A C C T C T T C A T T T G C A C T  
G A C C A C C T C T T C A T T T G C A C T G A C C A A C T T T T C A T T T G C A C T G A C C A T C T C T T C A T T T G C A C T G A C C A A C T T T T C A T T T G C A A T T C T G G C A A T G A  
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T C A A T A G T T G A T A A A A A T T A C T A A C C C C T T A G A A A G T T T C A G A C C G T C T A A C G T G G A A C A T C G C G G A G A C C C A T T T G T T T C G G A A A T T G C A C C G T  
G A G T G A T T T G C A C C T A A T T G G T T A T T T T A A T A A T C A T T A A A T T A T A G A C G C C C A A T T C G G A A G C C G A A A A G A A C C G C C C G C A C G T G C G C A G A  
A G C T C G A A G A G C A A C G T G T C A A A A C C C A T T C C A C A T C T T C A C C A C A A C T C G C T C A T T T T G A A A C A A G G A T A T T T G G A A A A G A A G C G A G G A T T G  
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A G A A A G G T A T G A A T T A C T G G A A G C C C C C C T C A C T G A G T T T C C A G C A A G T T C A G A G T T T T T A T T G G A A T T T T T G C C A A T T T T C A T T A G A C T T T A  
G A G C C T A T T G C T A T T T T G T G G A C A G G T T T A A A C A T T T T C A A A A A A A A A T T G A G A A A T G T C T G A A A A A T T T G G A G T G T G A C A G T T T T C T G A A T T T  
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T T G T C A A A A T A T T T T T T T G G A C A A T C T A G A T T C T G A A A A T T T T C A A A A A A A G A T A A T C T C T A A A C A A A C T A A A T T C A A A A T G T T C T A A A G G T  
T C T T T A T T T T C C A T G C A A C T C T A A A A T C T T C C C G T A T A T T T T T T G G A A A G T C T T A T G A T G T T T A G A C G G T T T A A A T T T T T G A T G A T T T A A A T T  
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C A C G C A A A T C T A T T A T T A T G G A C C A C C C A A C T T A G A A C A C C T T C A A T A C T T C T T T T C T G T T C A A A A A A T G A T C A A C T T G C T G A A A A A A A T T T  
T T T G T A G G A A A T G A T G C G T G A A C A G A A G G C G T G C G C C G A A A C A A G A A A A G G A G A A A A G C G C T A A A A G C C G A G C A A G T G A G C A A G A A G C  
T T T C A A T G C A A A T G G A C A A G A A G T C G C C T T G A A G G C T C A C C T C C C T T C T A C T C C C C A C A A A A T C A C C A T C A A A C A A A T C A C A C T T T T G T A T C A T T  
T T G C G T C C

Fig. 35B

MEDLTPNTSLDTTTTNNDTTS DREAAPTTLNLTPTASESENSLSPVTAEDLIAKSIKEGCPKRTSND FMFLQSMGEG  
 AYSQVFRCREVATDAMFAVKVLQKSYLNRHQMDAIIREKNILTYLSQECGGHPFVTQLYTHFHDQARIYFVIGLV  
 ENGD LGESLCHFGSFDMLTSKFFASEILTGLQFLHDNKIVHRDMKPDNVL IQDGHILITDFGSAQAFGGLQLSQEGFT  
 DANQASSRSSDSGSPPPTRFYSD EEEENTARRTT FVG TALYVSP EMLADGDVGPQTDI WGLGCILFQCLAGQPPFRAV  
 NQYHLLKRIQELDFS FPEGFP EEASEIIAKILVRDPSTRITSQELMAHKFFENVDWVN IANIKPPVLHAYIPATFGEP  
 EYYSNIGPVEPGLDDRALFRLMNLGNDASASQPSTPSNVEHRGDPFVSEIAPRANSEAEKNRAARAQKLEEQRVK  
 NPFHIFTNNSLILKQGYLEKKRGLFARRRMFLLTEGPHLLYIDV PNLVLKGEVPWTPCMQVELKNSGTFFIHTPNR  
 VYYLFDLEKKADEWCKAINDVRKRYSVTIEKTFNSAMRDGTFGSIYGKKKSRKEMMREQKALRRKQEKEKKAL  
 KAEQVSKKLSMQMDKKSP

Fig. 36

MEDLTPNTSLDTTTTNNDTTS DREAAPTTLNLTPTASESENSLSPVTAEDLIAKSIKEGCPKRTSND FMFLQSMGEG  
 AYSQVFRCREVATDAMFAVKVLQKSYLNRHQMDAIIREKNILTYLSQECGGHPFVTQLYTHFHDQARIYFVIGLV  
 ENGD LGESLCHFGSFDMLTSKFFASEILTGLQFLHDNKIVHRDMKPDNVL IQDGHILITDFGSAQAFGGLQLSQEGFT  
 DANQASSRSSDSGSPPPTRFYSD EEVPEENTARRTT FVG TALYVSP EMLADGDVGPQTDI WGLGCILFQCLAGQPPFR  
 AVNQYHLLKRIQELDFS FPEGFP EEASEIIAKILVRDPSTRITSQELMAHKFFENVDWVN IANIKPPVLHAYIPATF  
 GEPEYYSNIGPVEPGLDDRALFRLMNLGNDASASQPSTFRPSNVEHRGDPFVSEIAPRANSEAEKNRAARAQKLEE  
 QRVKNPFHIFTNNSLILKQGYLEKKRGLFARRRMFLLTEGPHLLYIDV PNLVLKGEVPWTPCMQVELKNSGTFFIH  
 TPNRVYYLFDLEKKADEWCKAINDVRKRYSVTIEKTFNSAMRDGTFGSIYGKKKSRKEMMREQKALRRKQEKEE  
 KKALKAEQVSKKLSMQMDKKSP

Fig. 37

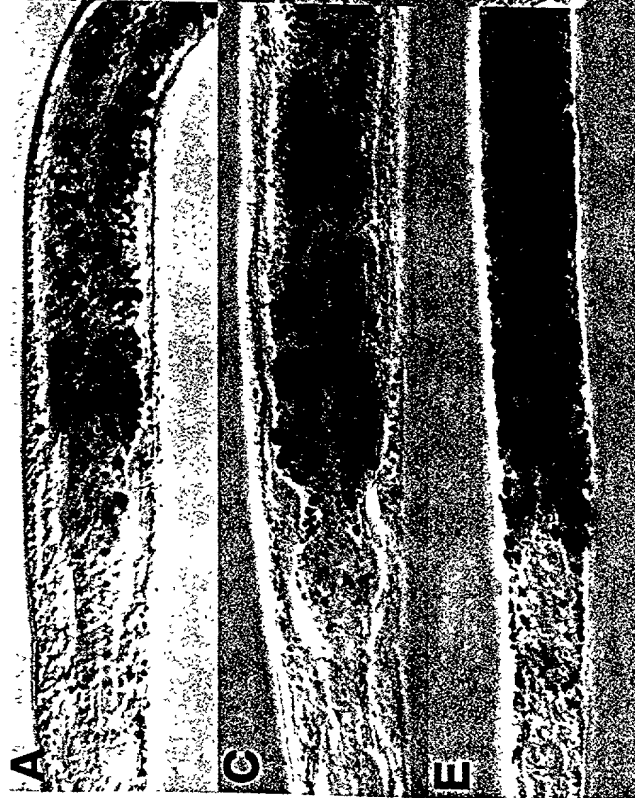


FIG. 38A

FIG. 38C

FIG. 38E

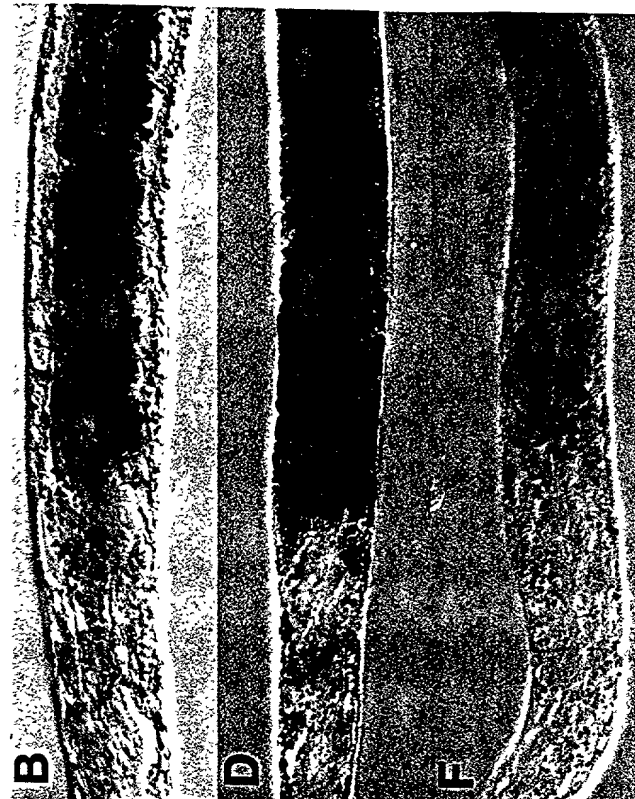


FIG. 38B

FIG. 38D

FIG. 38F

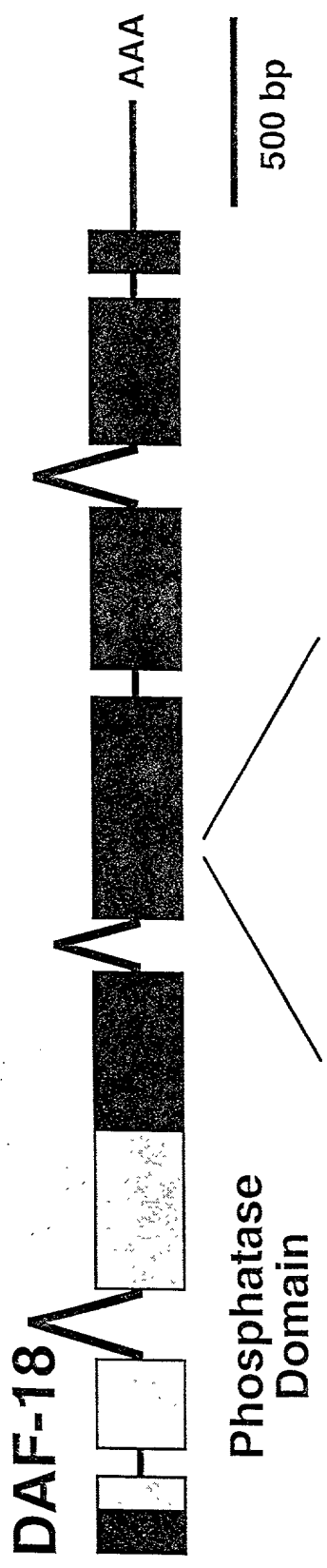


FIG. 39A

e1375

570

Q A L T Q M N P K

caagcgttgactcaa atgaatccaaaa

578

caagcgttgactcaatgcgttgactcgttgacgaatccaaaa

Q A L T Q C V D S M R \*

DAF-18 48 **IFRTAVSSNR** CRTEYQNIIDL **DCATITDRII** AIGYPATGIE ANFRNSKVQT

PTEN 4 **ILKEIVSRNK** RRYQEDGFDL **DLTYIYPNII** AMGPAERIE GVIYRNINDDV

DAF-18 98 **QCETTRRHGK** GNVKVFNLRG GYYTDADNFD GNVICFDMTD **HHPPSTELMA**

PTEN 54 **VRFLDSKH.K** NHYKLYNLCA ERHYDTAKEN CRVAQYFFED **HNPEQTELIK**

DAF-18 148 **PFCREAKEWL** **EADDKHVIAV** HCKAGKGRTG **WICAILIYI** NFYSPRQIL

PTEN 103 **PFCEDLDQWL** SEDDNHVAAI HCKAGKGRTG **WICAILLHR** GKFLKAQEA

DAF-18 198 **DYTSIIRTKN** NKGVITPSQR RYIYTHKLR ERRLNILELR **MLIGVYVER**

PTEN 153 **DFYGEVKTRD** KKGVTIPSQR RYVYYSYLL **KNHLDYREVA** ILFHKMMFET

DAF-18 248 **PKTWGGGSK** IKVEVNGNST ILFKPD.**PL** IISKSNHQRE **RATWNNCDT**

PTEN 203 **IFMFSGGTCN** PQFVVCQLKV KIYSSNSGPT **RREDKFMYPE** **FPQRPVCGD**

FIG. 39B

## DAF-18 Protein

MVTPPPDVPSTSTRSMARDLQENPNRQPGEPVSEPYHNSIVERIRHIFRTAVSSNRCRTEYQNIDLDCAYITDRIIAIG  
YPATGIEANFRNSKVQTQQFLTRRHGKGNVVFNLGGYYDADNFDGNVICFDMTDHPPSLELMAPFCREAKEWLEAD  
DKHVIAVHCKAGKGRGTGVMICALLIYINFYPSPRQILDYYSIIRTKNNKGVTI PSQRRYIYYHKLRELERLNYLPLRMQL  
IGVYVERPPKTWGGGSKIKVEVGNSTILFKPDPLIISKSNHQERATWLNCDTPNEFDTGEQKYHGFSKRAYCFMVP  
EDAPVFVEGDVRIDIREIGFLKKFSDGKIGHVWFNTMFACDGGGLNGGHFEYVDKTQPYIGDDTSIGRKNGMRRNETPMRK  
IDPETGNEFESPWQIVNPPGLEKHITTEEQAMENYTNYGMI PPRTYISKILHEKHEKGIVKDDYNDRLPMGDKSYTESGK  
SGDIRGVGGPFEPYKAEHVLTFFVYEMDRALKSKDLNNGMKLHVLRCDTRDSKMMKSEVFGNLAHFNSTRRLQA  
LTQMNPKWRPEPCAFGSKGAEMHYPPSVRYSSNDGKYNGACSENLVSDFFEHNRNIAVLNRYCRYFYKQSTSRSRYPKRF  
RYCPLIKKHFIYPADTDDVDENGQPFHSPHYIKEQEKIDA EKAAGIENTGPSTSGSSAPGTIKKTEASQSDKVKPAT  
EDELPPARLPDNRVRRFPVVGVD FENPEEESCEHKTVESIAGFEPLHLFPHESYHPNTAGNMLRQDYHTDSEVKIAEQEAK  
AFVDQLLNGQGV LQEFMKQFKVPSDNSFADYVTGQAEVFKAQIALLEQSED FQRVQANAEVDLEHTLGEAFERFGHVVE  
ESNGSSKNPKALKTREQMVKETGKDTQKTRNHVLLHLEANHRVQIERRETCP ELHPEDKIPRIAHFSSENSFSDSNFDQAI  
YL

FIG. 40A

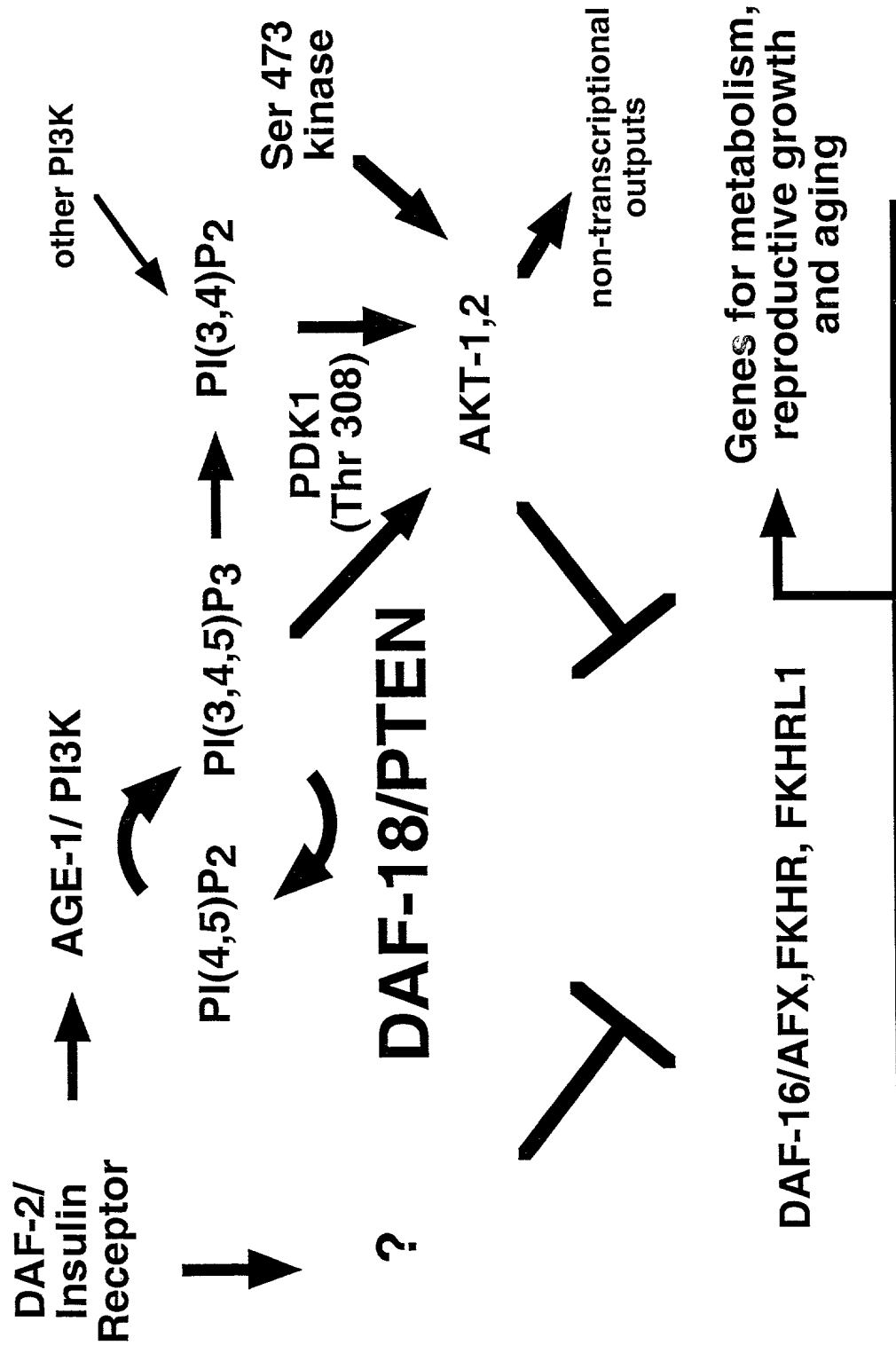
1 ttccaggtac atctactaac ccccaatggt tactctctct ccagatgtgc caagcacatc  
 61 gaccaggtcg atggctcgtg accttcaaga gaatccaaac cgacaacctg gtgaaccacg  
 121 tgtgtctgaa ccgtatcaca attcaatcgt cgagcggatt cgccatattt ttcggacggc  
 181 tgtatcttcc aatcgttgtc gcaccgagta ccaaaatata gacctagatt gtgcataat  
 241 cacagaccga atcatagcta tcggttatcc agcaacagga atcgaagcga atttccgtaa  
 301 ctcaaaagtt caaactcaac aattttctgac caggcggcac ggaaagggca acgtgaagg  
 361 gtttaacctg cgcggtggat actactacga tgcggataac ttcgatggaa atgttatttg  
 421 cttcgatatg actgatcata atccgcccag tctcgaatta atggctccgt tttgcagaga  
 481 ggctaaggaa tggcttgaag cagacgataa acatgtaata gctgtacact gtaaagctgg  
 541 aaaaggccgt accggagtga tgatatgtgc tcttctcata tacatcaact tctatccgag  
 601 cccacgacaa attctcgact actactcaat aattcgtaca aaaaacaaca aagggtgtcac  
 661 aattccatca caacgacgct acatttacta ctaccataag cttcgtgaac gtgagctcaa  
 721 ctatttacca ttgagaatgc agttgattgg tgtctacgtg gaacggccctc caaagacatg  
 781 ggggtggtggt tcaaaagataa aagtggagggt tggaaatggc tcgacaattt tatttaagcc  
 841 ggatcctctc ataactctca aatcaaatca tcagcgagag cgtgcgacgt ggctgaacaa  
 901 ctgtgatacg cctaacgaat tcgacaccgg agagcaaaaa tatcatggat ttgtttccaa  
 961 gagagcatac tgttttatgg tgccagaaga tgctccagta tttgtcgaag gagatgttcg  
 1021 tatagacatt cgcgaaatcg gatttctcaa aaagttttcg gacgggaaga ttgggtcatgt  
 1081 ttggttcaat acaatgttcg catgtgatgg aggactcaac ggtggacatt tcgagtacgt  
 1141 agacaaaact cagccgtaca tcggagacga tacatcaatc ggacggaaaa atggaatgcg  
 1201 aagaaatgaa acgcccgtgc gaaaaattga tccagaaact ggaaatgaat ttgagtctcc  
 1261 gtggcaaata gtgaatcctc ctggactgga aaaacatatt acggaggaac aagcaatgga  
 1321 aaattatacc aattatggca tgattcctcc tcgatacacg atcagcaaga ttcttcacga  
 1381 aaagcatgaa aaaggatcgc tcaaggatga ctataatgat cgtaagctgc caatgggaga  
 1441 caaatcatac acggaatcag gaaaaagtgg agatattcga ggagtcggtg gtccatttga  
 1501 gataccatat aaagctgagg aacatgttct cacatttcca gtttatgaaa tggatcgagc  
 1561 attgaagagt aaagatctta acaacggaat gaaacttcac gttgttcttc gttgtgtaga  
 1621 tactcgtgat tcaaaaatga tggaaaagag cgaagtgttc ggcaatctgg cattccataa  
 1681 tgaatcgaca cggaggcttc aagcgttgac tcaaatgaat ccaaatggc gacctgaacc  
 1741 gtgtgcgttc ggatccaaag gtgctgaaat gcattaccct ccgtcgggtc gatattcaag  
 1801 caatgatgga aagtataatg gagcctgcag tgagaacctt gttagcgatt ttttcgagca  
 1861 cagaaatatt gccgttctta atcgatattg ccgatatttc tacaagcaac gcagtacatc  
 1921 tcgaagccgt tatccaagaa aattcagata ctgtcctctg atcaagaaac atttctacat  
 1981 tccagctgat accgatgatg ttgatgaaaa tgggcaaccg ttcttccact caccagagca  
 2041 ttacattaaa gaacaggaaa aaatagacgc agagaaaagca gctaaaggaa ttgaaaatac  
 2101 tggacccagt acttcaggat caagtgtctc cggaactatc aagaaaacgg aagcttcaca  
 2161 atccgacaag gtgaagccgg caactgaaga cgaacttcct cctgcgaggg taccggataa  
 2221 tgtgcgaaga tttccagtcg tcggcgttga tttcgaaaat ccggaagaag aatcggtgga  
 2281 acacaaaacc gtagagtcaa tagctggttt tgaaccactc gaacatctat tccatgaatc  
 2341 ataccatcca aatacgcccg gtaacatgct gcgtcaggat tatcacactg attcgggaag  
 2401 gaaaatagct gaacaagagg caaaagcctt cgttgaccag ttgcttaatg gacaagggtg  
 2461 attacaagag tttatgaagc aattcaaagt accatcggac aattcctttg ctgattatgt  
 2521 aaccggacag gccgaagttt ttaaagcaca gattgcgtta ctggagcagt cggaggattt  
 2581 tcaacgagtt caagcgaatg cagaggaagt cgatcttgaa cacactcttg gtgaagcgtt  
 2641 tgagcgattc gggcacgttg tagaagaatc gaatggttct tctaaaaatc caaaagccct  
 2701 gaaaactcga gaacaaatgg tgaaagaaac tggcaaagac actcagaaga cccgcaatca  
 2761 tgtgcttcta catttggaag ctaatcatcg tgtgcaaata gagcgtcgtg aaacgtgccc

FIG. 40B

2821 ggagctacat ccagaggata aaatcccaag aattgctcat ttttccgaaa acagcttctc  
2881 ggattcgaat tttgatcaag ctatttattt gtaaaccctaa aacaaaactt ttagaagatt  
2941 ttcttcttac tgaccctcca attttcagat aatttcaatg ttttaagttt tctcttcaaa  
3001 gtatcattca ctttctgtat agtgttttgt tttttaacaa actattgttc gattattttg  
3061 tatattcata ttatagctct caacttcccg attttccacg tatatatgta tattttgccg  
3121 ggtgaaaaat agcaattccc tatgaatgta tccccctcca tctgttttct tactcagaaa  
3181 ttgtaattca cattgcgggt catcactaat cctatgggct ttaacacaat tctcccataa  
3241 attaattgta cttaccaatt ttttgtttaa ttatttagat ttgtaacatt gaaattggtg  
3301 ataa

FIG. 40B

FIG. 41



	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2
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atg aag ttt caa tac tac tcg aag aaa gct gct gga aag aca atg tct aat agt gtc tcc  
M K F Q Y Y S K K A A G K T M S N S V S

atg tcc agt gac aat cgc atg gag gat ttt aaa cgt cgt ttt cgt cga agt gga tcg tta  
M S S D N R M E D F K R R F R R S G S L

gga att cca ttt gtc cca gaa gaa gat gtt aaa caa ctc ttc aca cca act cgt act gtt  
G I P F V P E E D V K Q L F T P T R T V

cgt cga gaa gca tct att cgc gaa ggg gat gag gaa gaa gga gta caa att ctc aca ata  
R R E A S I R E G D E E E G V Q I L T I

att gtc aag tca agt cgt gtt tog gag gat atc tca aaa atg att gca aac ctc cct gat  
I V K S S R V S E D I S K M I A N L P D

cac act cgt atc aaa cat ttg gag act cgt gac agt caa gat gga agt tcc aaa act atg  
 H T R I K H L E T R D S Q D G S S K T M

gat gtt ctt cta gag att gag ctc ttt cat tat gga aaa caa gaa gca atg gat ctt atg  
D V L L E I E L F H Y G K Q E A M D L M

aga ctt aat ggg ctt gat gtt cat gag gtg tca tcg act att cgt cca act gca ata aaa  
R L N G L D V H E V S S T I R P T A I K

gag caa tat aca gag cct gga tct gat gat gcg aca acc ggt tct gaa tgg ttt cca aaa  
E Q Y T E P G S D D A T T G S E W F P K

agt att tat gat ttg gat att tgt gca aaa aga gtg att atg tat gga gca ggg ctg gac  
S I Y D L D I C A K R V I M Y G A G L D

gct gat cat cct ggt ttc aaa gat acc gag tat cgt caa cgt cga atg atg ttt gct gaa  
A D H P G F K D T E Y R Q R R M M F A E

ctg gcg ctc aat tac aaa cac ggt gag cca att ccg cga acc gaa tat aca tca tcc gaa  
L A L N Y K H G E P I P R T E Y T S S E

cgg aaa act tgg gga att ata tat aga aaa ttg aga gaa ttg cac aaa aag cac gca tgc  
R K T W G I I Y R K L R E L H K K H A C

aag cag ttt ctt gat aac ttt gag cta ctg gag aga cat tgt gga tac tcg gaa aat aat  
K Q F L D N F E L L E R H C G Y S E N N

att ccg caa cta gaa gat atc tgc aag ttt ttg aaa gca aaa act gga ttc cgt gtt cgc  
I P Q L E D I C K F L K A K T G F R V R

FIG. 42

cca gtc gcc gga tac tta tca gct cgt gat ttc ttg gca ggt ctt gca tat cgt gtc ttc  
P V A G Y L S A R D F L A G L A Y R V F

ttc tgc act caa tac gtt cgc cat cat gcc gat cca ttt tac act cca gaa cca gac acc  
F C T Q Y V R H H A D P F Y T P E P D T

ggt cac gag ctc atg ggt cac atg gct cta ttc gct gat cca gat ttt gct cag ttt tct  
V H E L M G H M A L F A D P D F A Q F S

caa gag att gga tta gct tct ctt gga gca tca gag gaa gat ttg aag aag ctt gca aca  
Q E I G L A S L G A S E E D L K K L A T

ctc tac ttc ttt tcc att gaa ttt ggt ctc tcg tct gat gac gct gcc gat tct cca gta  
L Y F F S I E F G L S S D D A A D S P V

aaa gaa aat gga tca aat cat gaa aga ttt aaa gta tac gga gca gga ctt ctg agc agt  
K E N G S N H E R F K V Y G A G L L S S

gct ggc gag ttg caa cat gcc gtt gag ggt agt gca acc att att cgt ttt gat ccg gat  
A G E L Q H A V E G S A T I I R F D P D

cgt gtt gtt gag caa gaa tgt ctc att act act ttc cag tca gcg tat ttc tat act aga  
R V V E Q E C L I T T F Q S A Y F Y T R

aat ttt gaa gag gcc cag cag aaa ctc aga atg ttc acc aac aac atg aaa cgt ccc ttc  
N F E E A Q Q K L R M F T N N M K R P F

att gtt cgt tac aac cca tac aca gaa agc gtc gaa gtt ctc aac aac tcc cgt tcc att  
I V R Y N P Y T E S V E V L N N S R S I

atg ttg gca gtg aac tct ctc cgc tca gac atc aac ctg ctc gcc gga gct ctc cac tac  
M L A V N S L R S D I N L L A G A L H Y

atc ctg tag  
I L \*

FIG. 42

attacccaagtttgaggtagcattgctctcttcaatcat

atg gat tgg ttg ttt cag atg gca tcc gca atg aag ttt caa tac tac tgg aag aaa gct  
M D S L F Q M A S A M K F Q Y Y S K K A

gct gga aag aca atg tct aat agt gtc aaa aac tgg att ccg tgt tgg ccc agt cgc cgg  
A G K T M S N S V K N W I P C S P S R R

ata ctt atc agc tgg tga ttt ctt ggc agg tct tgc ata tgg tgt ctt ctt ctg cac tca  
I L I S S \*

ata cgt tgg cca tca tgc cga tcc att tta cac tcc aga acc aga cac cgt tca cga gct  
cat ggg tca cat ggc tct att cgc tga tcc aga ttt tgc tca gtt ttc tca aga gat tgg  
att agc ttc tct tgg agc atc aga gga aga ttt gaa gaa gct tgc aac act cta ctt ctt  
ttc cat tga att tgg tct ctc gtc tga tga cgc tgc cga ttc tcc agt aaa aga aaa tgg  
atc aaa tca tga aag att taa agt ata cgg agc agg act tct gag cag tgc tgg cga gtt  
gca aca tgc cgt tga ggg tag tgc aac cat tat tgg ttt tga tcc gga tgg tgt tgt tga  
gca aga atg tct cat tac tac ttt cca gtc agc gta ttt cta tac tag aaa ttt tga aga  
ggc cca gca gaa act cag aat gtt cac caa caa cat gaa acg tcc ctt cat tgt tgg tta  
caa ccc ata cac aga aag cgt cga agt tct caa caa ctc ccg ttc cat tat gtt ggc agt  
gaa ctc tct ccg ctc aga cat caa cct gct cgc cgg agc tct cca cta cat cct gta g

FIG. 43

FIG. 44A

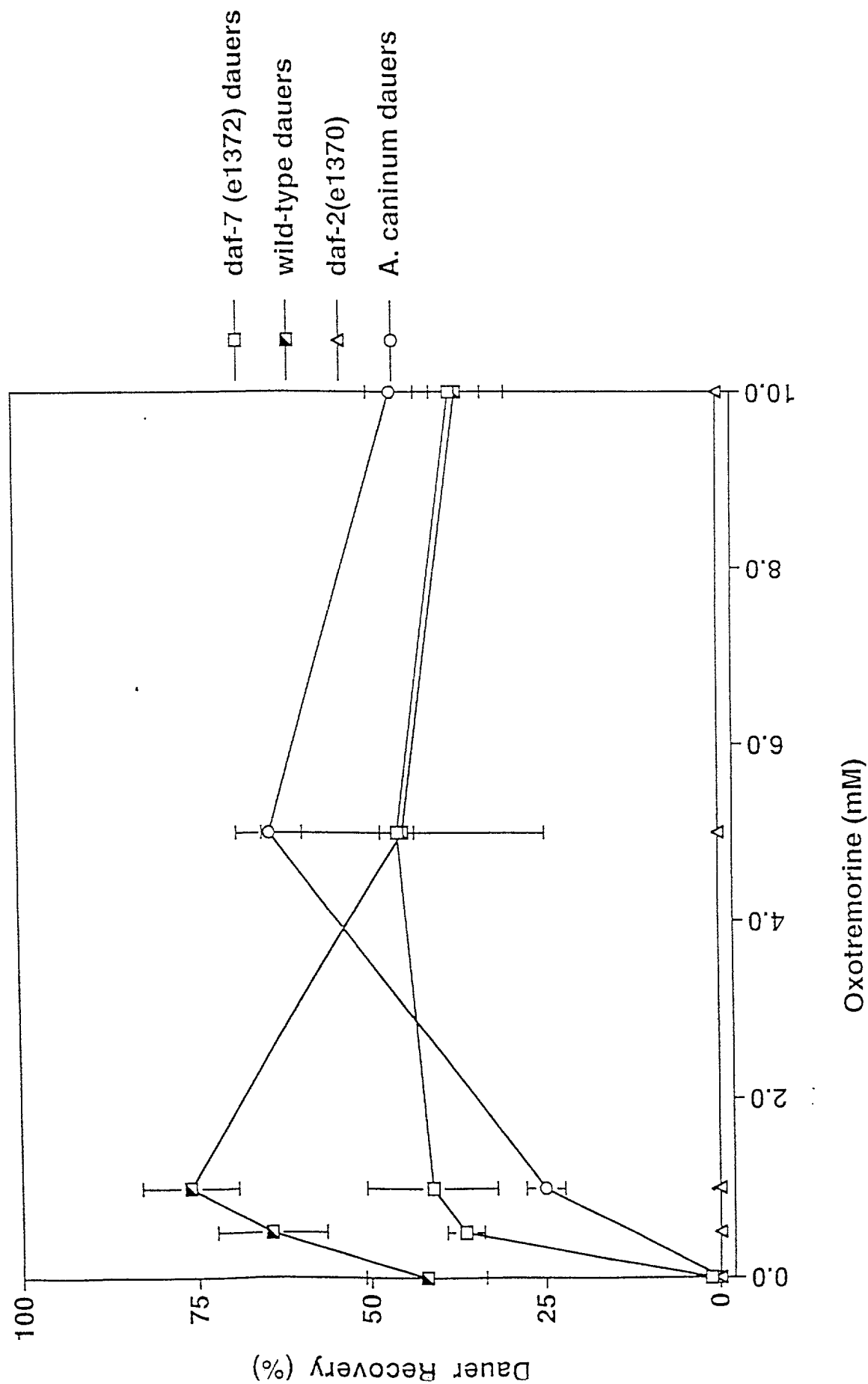


FIG. 44B

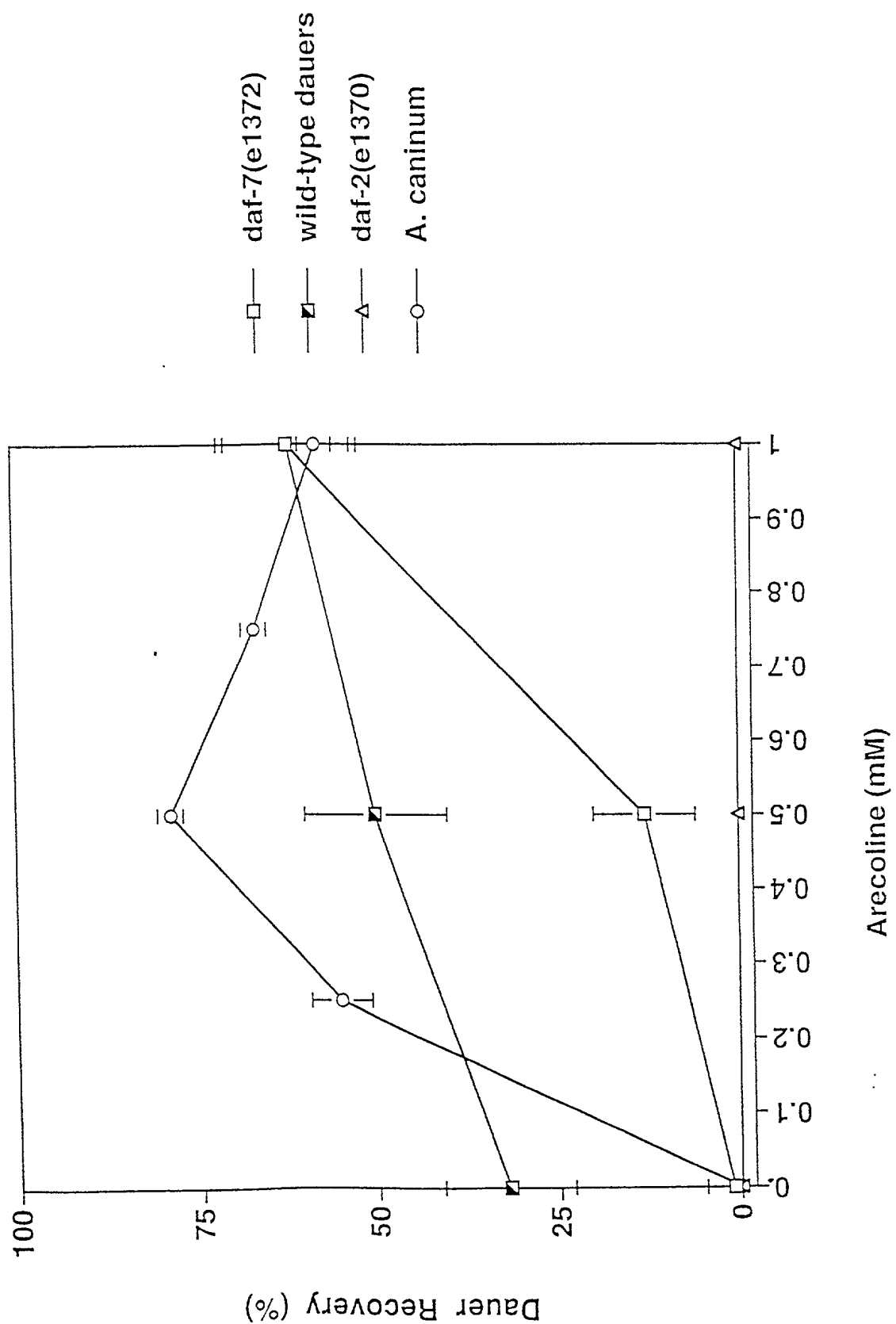


FIG. 44C

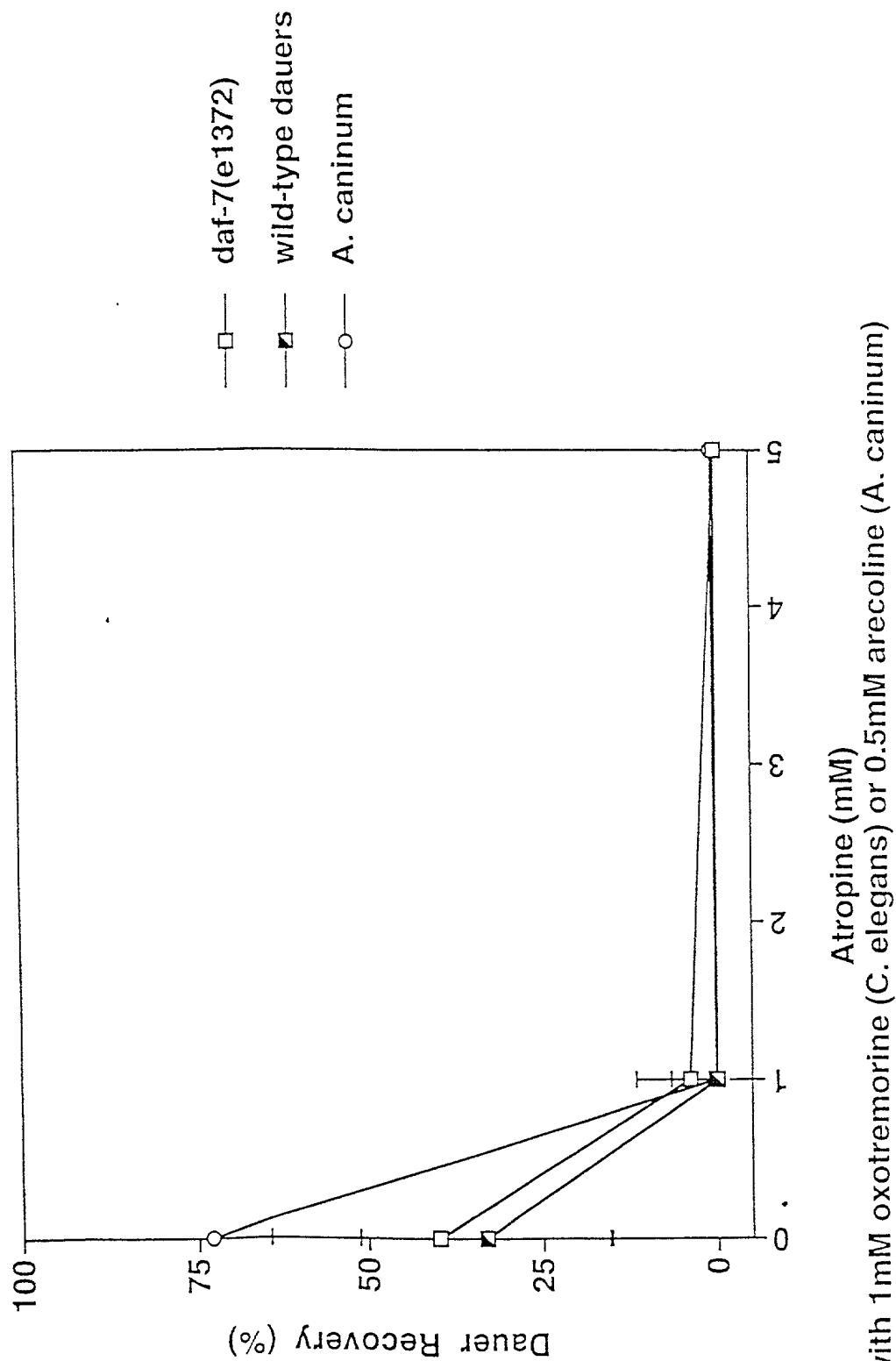


FIG. 45A

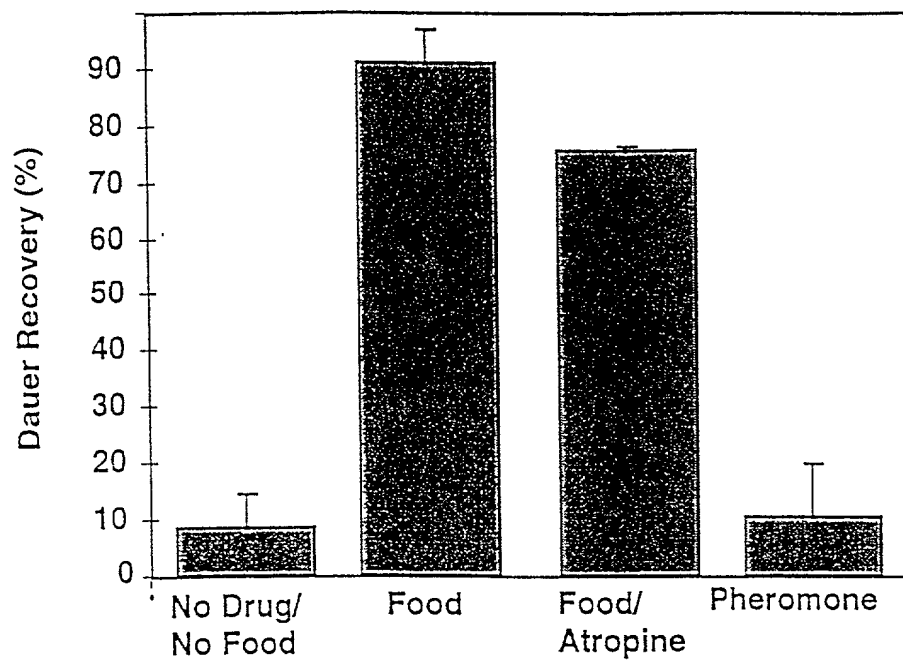


FIG. 45B

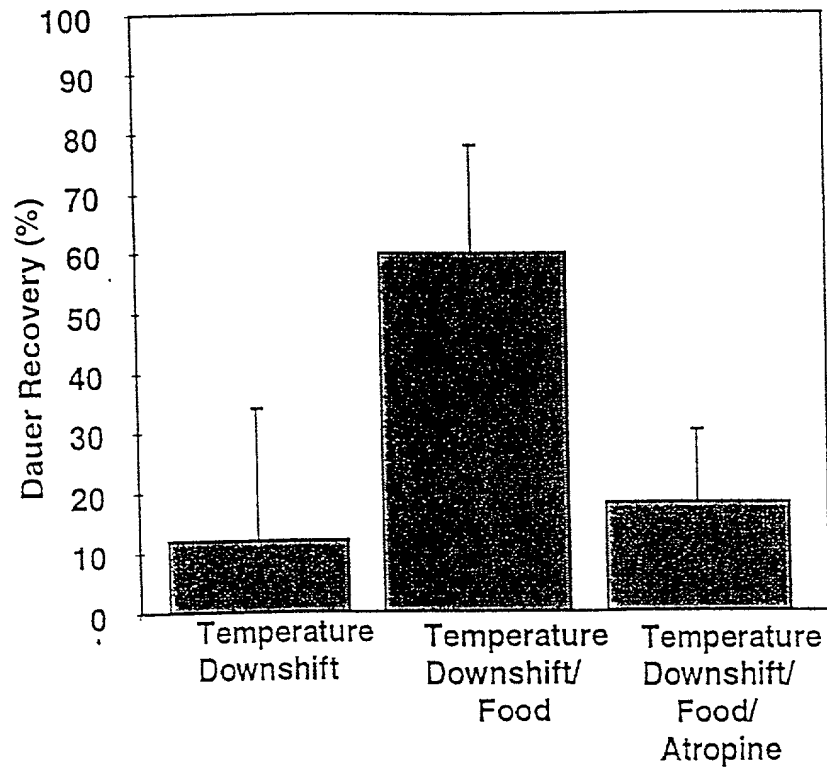
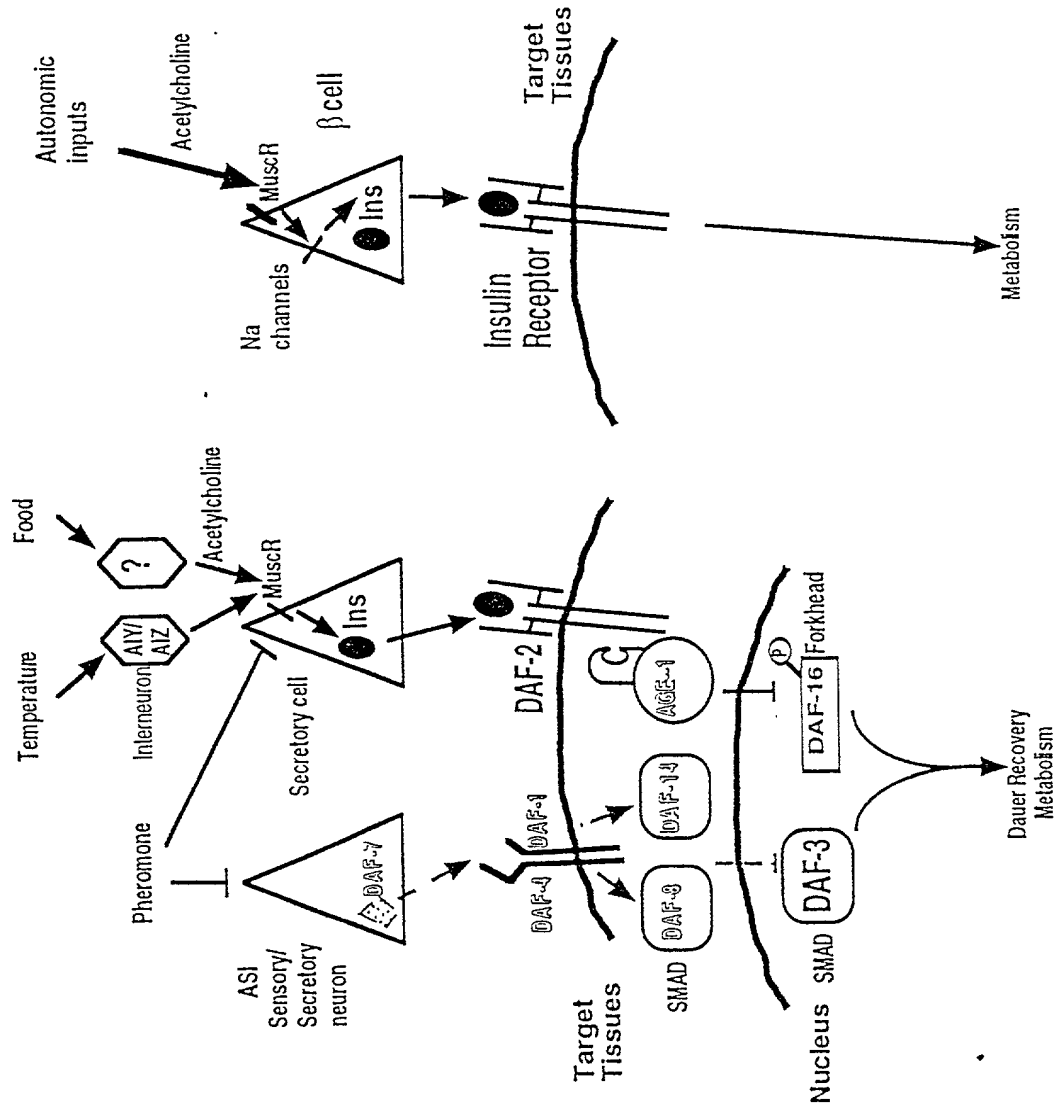


FIG. 46

*C. elegans*

Mammals



ATTCGGCATGAGCATGGaGCTTCGAGTCCTAGAGAACACAAAACGTTCCCGGCGGAACCTGGGtCTGGACTGCGAC  
GAGACTCAAGCGAGTCCCGCTGCTGCCGATATCCCCTCACAGTGGACTTTGAGGCTTTCCGGCTGGGACTGGATCAT  
CGCACCTAAGCGCTACAAGGCCAACTACTGCTCCGGCCAGTGGGAGTACATGTTTCATGCAAAAATATCCGCATACC  
CATTTGGTGCAGCAGGCCAATCCAAGAGGTTATGcTGGGCCCTGTTGTACCCCCACCAAGATGTCCCCAATcAACA  
TgcTctACTTCAATGACAAGCAGCAGATTATcTACGGCAAGATCCCTGGCATGGTGGTGGATCGCTGTGGcTGCTC  
TTAAGGTGGGGGATAGAGGATGCCTCCCCACAGACCGTACCCAAGACCCATAGCCcTGCCCAATCCACCGCCTG  
ATCCAAACAT

FIG. 47A

IRHEHGASSPREHKTFPAEPGSLRRDSSSRCCRYPLTVDFEAFGWDWIIAPKRYKANYCSGQWEYMFQMOKYPHT  
HLVQQANPRGYAGPCCTPTKMSPINMLYFNDKQQIIYGKIPLAMVVDRCGCS

FIG. 47B

**COMBINED DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled THERAPEUTIC AND DIAGNOSTIC TOOLS FOR IMPAIRED GLUCOSE TOLERANCE CONDITIONS, the specification of which

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as Application Serial No. \_\_\_\_\_  
and was amended on \_\_\_\_\_.

☐ was described and claimed in PCT International Application No. \_\_\_\_\_  
filed on \_\_\_\_\_ and as amended under PCT Article 19 on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Serial Number	Filing Date	Priority Claimed?
PCT	PCT/US98/10080	May 15, 1998	Yes
			Yes/No
			Yes/No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

### COMBINED DECLARATION AND POWER OF ATTORNEY

Serial Number	Filing Date	Status
08/888,534	July 7, 1997	Pending
08/857,076	May 15, 1997	Pending

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Paul T. Clark, Reg. No. 30,162, Karen L. Elbing, Ph.D. Reg. No. 35,238, Kristina Bieker-Brady, Ph.D. Reg. No. 39,109, Susan M. Michaud, Ph.D. Reg. No. 42,885.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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Signature:			Date: